

Viral ion channel disruption of the somatotroph population in transgenic mice

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ABSTRACT

Spontaneous and induced murine genetic models of disrupted somatotroph function have been useful in elucidating their relationships with other pituitary cell types as well as in studying downstream effects of GH deficiency. To disrupt somatotroph cell function in a temporally controlled fashion, I have applied a transgenic ion-channel conditional ablation strategy. The H37A variant of the influenza virus M2 protein that conducts monovalent cations unless blocked with the anti-influenza drug Rimantadine. This channel, previously shown to ablate GHRH neurons, has been targeted to somatotrophs using the hGH locus control region to attempt the conditional ablation of this cell population. Three fertile GH-M2 mouse lines have been generated with a growth phenotype and GH, IGF-1, PRL and TSH deficiencies that vary with copy number. Treatment of low and medium copy line male mice with GHRH and GHRP-6 suggests a greater effect on the low copy line than the hormone levels may indicate. It has not been possible to reverse the GH deficiency with Rimantadine treatment *in vivo*. A second model of drug-inducible somatotroph silencing using Ivermectin-sensitive chloride channels was therefore also investigated in preliminary studies.

GH-M2 mice have been crossed with the GH-GFP and PRL-DsRed transgenic mouse lines to image the effect M2 expression has had on the GH and PRL cell networks with 2-photon microscopy. Analysis shows that the somatotroph network is disrupted, to varying degrees with age and GH deficiency, in GH-M2 mice. Unlike the GHRH-M2 pituitary, however, clusters are still observed.

Counterstaining the blood vessels of a low copy line pituitary with rhodamine indicates that the majority of the remaining GH cells are in contact with the capillary system.

The GH-M2 mice provide models for studies of the origin, maintenance and interrelationships of the pituitary cell types.

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ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AVP	arginine vasopressin
BMP	bone morphogenic protein
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
CFP	cyan fluorescent protein
CMV	cytomegalovirus
hCMV	human cytomegalovirus
CPHD	combined pituitary hormone deficiency
CRE	cAMP response element
CREB	cAMP response element binding protein
Cyp	cytochrome p450
DAPI	4',6-diamidino-2-phenylindole
DIG	digoxigenin
DMEM	Dulbecco's minimal essential medium
DT-A	diphtheria toxin subunit A
DTR	diphtheria toxin receptor
iDTR	inducible diphtheria toxin receptor
DTT	dithiothreitol
ER	estrogen receptor
ER ^T	tamoxifen-inducible estrogen receptor
FACS	fluorescent activated cell sorting
FGF	fibroblast growth factor
bFGF	basic fibroblast growth factor
FGFR	fibroblast growth factor receptor
FIAU	1-(2-deoxy-2-fluoro-b-d-arabinofuranosyl)-5-iodouracil
FKBP	FK506 binding protein
FRB	FKBP/rapamycin binding protein

FRET	fluorescence resonance energy transfer
FRT	Flp recombinase target
FS	folliculostellate cell
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
GH	growth hormone
hGH	human growth hormone
rGH	rat growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
GHRP	growth hormone releasing peptide
GHS-R	growth hormone secretagogue receptor
GluCl	glutamate-gated chloride channel
GPCR	G-protein coupled receptor
GSK	glycogen synthase kinase
HB-EGF	heparin-binding epidermal growth factor-like growth factor
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HRE	hypoxia-responsive element
HS	hypersensitive site
HSV	herpes simplex virus
I3C	indole-3-carbinol
IGF	Institut de Génomique Fonctionnelle
IGF-1	insulin-like growth factor-1
IGHD	isolated growth hormone deficiency
IRES	internal ribosome entry site
IRS	insulin receptor substrate
IVF	<i>in vitro</i> fertilisation
IVM	Ivermectin
JAK	Janus kinase
LC	low copy
LCR	locus control region

LH	luteinising hormone
MAPK	mitogen-activated protein kinase
MC	medium copy
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MUP	major urinary protein
NACWO	Named Animal Care and Welfare Officer
NAD	nicotinamide adenine dinucleotide
N-CAM	neural cell adhesion molecule
N-CoR	nuclear corepressor
NHPP	National Hormone and Peptide Program
NT	non-transgenic
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFA	paraformaldehyde
PI	phosphatidyl inositol
Pit-1	pituitary specific transcription factor
PKA	protein kinase A
hPLAP	human placental alkaline phosphatase
POMC	pro-opiomelanocortin
PRL	prolactin
PRLR	prolactin receptor
Prop-1	prophet of Pit-1
PVA	polyvinyl alcohol
RASSL	receptor activated solely by synthetic ligands
RIA	radioimmunoassay
RIPA	radioimmunoprecipitation buffer
RNAi	ribonucleic acid interference
RNase	ribonuclease
RNP	ribonucleoprotein

rt-PCR	reverse transcriptase PCR
rtTA	reverse transcriptional transactivator
SDS	sodium lauryl sulphate
SEM	standard error of the mean
SSC	saline sodium citrate
ssDNA	single stranded deoxyribonucleic acid
SST	somatostatin
Stat	signal transducer and activator of transcription
T	transgenic
T3	triiodothyronine
T4	thyroxine
TBE	tris-borate-EDTA buffer
TBS	tris buffered saline
TE	tris-EDTA buffer
Tet	Tetracycline
TGF	transforming growth factor
Tgr	transgenic growth retarded
TH	thyroid hormone
TIDA	tuberoinfundibular dopaminergic (neuron)
TIRF	total internal reflection fluorescence
TK	thymidine kinase
TNF	tumour necrosis factor
TRH	thyrotropin releasing hormone
TSH	thyroid stimulating hormone
tTA	transcriptional transactivator
TUNEL	terminal deoxynucleotidyl transferase Biotin-dUTP nick end labelling
UTR	untranslated region
VIP	vasointestinal peptide
YFP	yellow fluorescent protein
Z/AP	lacZ/human placental alkaline phosphatase

1. GENERAL INTRODUCTION

The hormones of the pituitary gland play varied and important roles in vertebrate growth and development and are responsible for coordinating the hormone response of the other endocrine organs. The following sections give a general introduction to the field. The aims of this thesis are given on page 43.

1.1 The Pituitary Gland

The pituitary is situated at the base of the brain, attached to the hypothalamus but is located outside the blood-brain barrier. It is divided into three distinct lobes - the posterior, intermediate and anterior lobes.

The posterior lobe is an extension of the hypothalamus and contains nerve termini that store and secrete the hormones oxytocin and arginine vasopressin (AVP). These hormones are produced in the nerve cell bodies located in the supraoptic and paraventricular nuclei of the hypothalamus and transported to nerve termini for storage and secretion.

α -melanocyte-stimulating hormone (α MSH), which is cleaved from the precursor protein pro-opiomelanocortin (POMC), is synthesised and secreted from specialised cells of the intermediate lobe.

The endocrine cells of the anterior lobe are responsible for the synthesis and secretion of several hormones: somatotrophs - growth hormone (GH),

lactotrophs - prolactin (PRL), thyrotrophs - thyroid stimulating hormone (TSH), corticotrophs - adrenocorticotrophic hormone (ACTH), gonadotrophs - luteinising hormone (LH) and follicle stimulating hormone (FSH). The hormones are stored within these cells in secretory vesicles ready for release upon stimulation. The synthesis and secretion of the hormones of the anterior pituitary is mainly controlled by hypothalamic factors. These molecules are produced in the cell bodies of neurons located in hypothalamic regions such as the arcuate and periventricular nuclei before being transported to the nerve termini in the median eminence of the hypothalamus. From here they are released into the hypophyseal portal system and carried to the endocrine cells of the anterior pituitary where they interact with cell surface receptors to initiate or inhibit secretion.

Non-endocrine cells are also located in the anterior pituitary. For example, folliculostellate cells are found throughout the anterior lobe. The function of these cells is as yet unknown but they are thought to have a role in local control of pituitary function by release of paracrine factors (Bilezikjian et al., 2003) and in long distance cell-cell communication within the anterior lobe (Fauquier et al., 2001).

1.2 Pituitary Development

Development of the pituitary is defined by specific spatial and temporal patterns of gene expression. The anterior and intermediate lobes of the pituitary develop from Rathke's pouch, a progressive invagination of the oral ectoderm

that begins in response to factors released from the diencephalon from e8.5 (Daikoku et al., 1982), such as BMP4 and FGF8 (Takuma et al., 1998). Several transcription factors are involved in development of Rathke's pouch, as studied by specific gene deletions. For example, homozygous *Hesx1* knockout mice (Dattani et al., 1998) had small, underdeveloped pituitaries that failed to make contact with the posterior lobe. Deletion of *Lhx3* (Sheng et al., 1996) or *Lhx4* (Sheng et al., 1997) results in formation of Rathke's pouch but the pituitary does not grow and develop further, and very few endocrine cells can be distinguished.

Figure 1.1 Schematic diagram of pituitary cell development (modified from Watkins-Chow and Camper, 1998). Progression of cell lineage development in the embryonic pituitary. Embryonic ages represent onset of transcription factor expression. A = ACTH, L = LH (and FSH), T = TSH, G = GH and P = PRL.

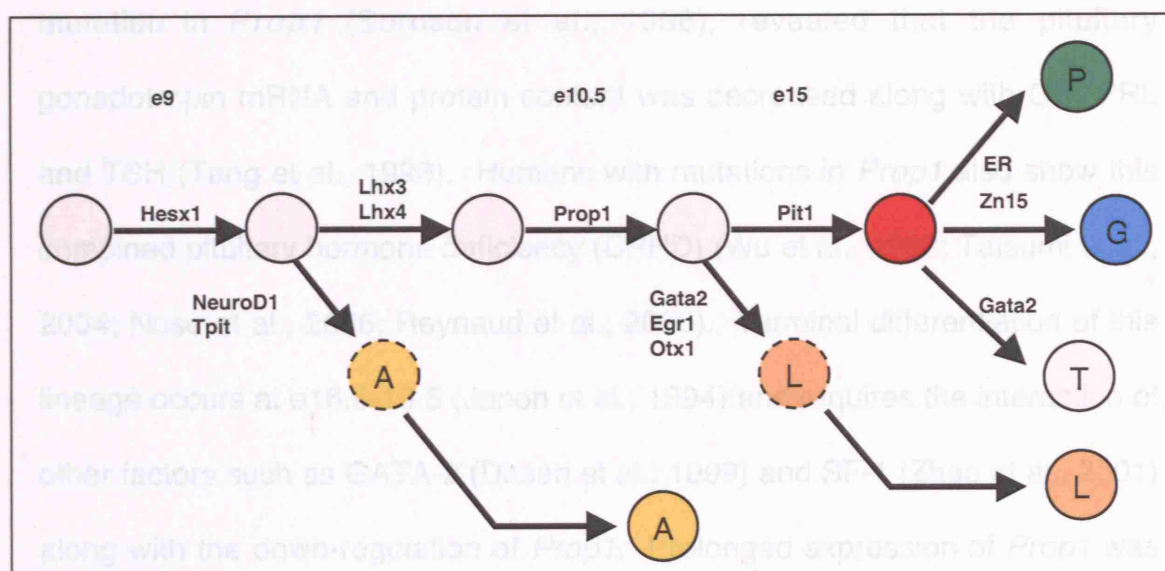


Figure 1.1 illustrates the progression of endocrine cell development in the anterior pituitary. Terminal differentiation of the endocrine cell lineages of the anterior pituitary begins at e12.5 when corticotroph cells expressing proopiomelanocortin (POMC) can be detected (Japon et al., 1994). The corticotroph cells are thought to differentiate from earlier HESX1 expressing precursor cells with a role for Hes1 and Notch activity in defining the pattern of corticotroph development (Zhu et al., 2006), but an exact mechanism has yet to be elucidated. Interaction between the corticotroph-specific transcription factor Tpit and Pitx-1 is required for activation of POMC transcription during anterior pituitary development (Lamolet et al., 2001; Pulichino et al., 2003).

Expression of the transcription factor Prophet of Pit1 (PROP1) is necessary for the differentiation of the gonadotroph cell lineage at e10.5 and progression of the developing mouse pituitary. Studies of the Ames dwarf, which carries a mutation in *Prop1* (Sornson et al., 1996), revealed that the pituitary gonadotropin mRNA and protein content was decreased along with GH, PRL and TSH (Tang et al., 1993). Humans with mutations in *Prop1* also show this combined pituitary hormone deficiency (CPHD) (Wu et al., 1998; Tatsumi et al., 2004; Nose et al., 2006; Reynaud et al., 2006). Terminal differentiation of this lineage occurs at e16.5-18.5 (Japon et al., 1994) and requires the interaction of other factors such as GATA-2 (Dasen et al., 1999) and SF-1 (Zhao et al., 2001) along with the down-regulation of *Prop1*. Prolonged expression of *Prop1* was shown to lead to a decrease in the number of terminally differentiated gonadotrophs during pituitary development in the embryonic and postnatal

mouse (Cushman et al., 2001; Vesper et al., 2006). PROP1 has a role in terminating *Hesx1* expression (Gage et al., 1996b) and is thought to have a role in initiation of *Pit1* expression (Sornson et al., 1996) to ensure progression of pituitary development. A decline in PROP1 expression coincides with initiation of *Pit1* (also known as POU1F1) expression at e15. Notch1 signalling is required to ensure progression and differentiation of the Pit-1 lineage (Zhu et al., 2006). In the absence of Notch1 Pit-1 fails to be initiated and the majority of the cells in the anterior pituitary differentiate into corticotrophs.

Somatotrophs, lactotrophs and thyrotrophs arise from a precursor cell type expressing PIT1 (Simmons et al., 1990) that differentiates to give rise to the three different cell types. This was confirmed by studies of the Snell dwarf mouse. These mice are characterised by an absence of somatotrophs, lactotrophs and thyrotrophs (Roux et al., 1982). A missense mutation in the *Pit1* gene is responsible for this defect (Li et al., 1990).

Several point mutations have been identified in human Pit-1 which led to a number of different disorders including CPHD and cretinism (Tatsumi et al., 1992). These mutations have been found to occur across the three binding domains of Pit-1 – the transactivation domain, POU-specific domain and POU-homeodomain (Ohta et al., 1992; Pfaffle et al., 1992; Radovick et al., 1992; Tatsumi et al., 1992; Turton et al., 2005). Both dominant and recessive forms have been found. In the dominant mutations, the mutant protein binds to DNA

but inhibits transcription possibly by preventing the wildtype protein from initiating activity (Radovick et al., 1992).

Other factors interact with Pit-1 to promote specific cell differentiation. For example, the zinc-finger protein Zn-15 binds synergistically with Pit-1 to the GH promoter to activate GH transcription (Lipkin et al., 1993), interaction of the transcription factor Gata-2 with Pit-1 activates the TSH- β subunit promoter (Gordon et al., 1997), and, the nuclear estrogen receptor (ER) regulates PRL gene transcription when Pit-1 is bound to the PRL enhancer (Day et al., 1990). Recruitment of repressor factors also affects specification of cell type. The nuclear receptor corepressor (N-CoR) is required for long term repression of the GH gene in lactotrophs (Scully et al., 2000). This repressor is recruited to the POU domain of Pit-1, along with other elements such as GH-1, to block Pit-1 induced GH transcription. *Pit-1* expression persists into adulthood unlike other transcription factors expressed during endocrine cell development (Ingraham et al., 1988).

1.3 The Pit-1 cell lineage

Somatotrophs, lactotrophs and thyrotrophs all differentiate from a Pit-1 expressing precursor cell during pituitary development. The common belief is that somatotrophs and lactotrophs are thought to differentiate from a common precursor cell, whereas thyrotrophs, although differentiating from a Pit-1 expressing precursor cell, form a separate developmental lineage from e14.5 (Japon et al., 1994).

1.3.1 Somatotrophs

Growth hormone is synthesised, stored and secreted from the somatotrophs of the anterior pituitary. This process is under strict regulation from stimulatory and inhibitory hypothalamic factors.

1.3.1.1 Growth hormone releasing hormone (GHRH)

The major stimulatory hypothalamic factor controlling GH synthesis and secretion is Growth Hormone Releasing Hormone (GHRH). GHRH was first isolated from patients with acromegaly caused by ectopic expression of this growth factor from pancreatic tumours (Guillemin et al., 1982; Rivier et al., 1982; Thorner et al., 1982). Application of this factor to primary cultured rat anterior pituitary cells resulted in an increase in GH secretion and somatotroph proliferation. It was found that this peptide had many similarities to an uncharacterised growth-hormone releasing factor found in the hypothalamus (Frohman et al., 1980). Immunocytochemistry of the hypothalamus, with an antibody raised to the synthetic pancreatic tumour peptide, was used to locate the majority of GHRH neuron cell bodies to the arcuate nucleus, with nerve terminals situated in the median eminence (Merchenthaler et al., 1984).

GHRH is known to act on somatotrophs through the GHRH receptor. Binding of GHRH to this G-protein coupled receptor activates the $G_{s\alpha}$ subunit which in turn stimulates an increase in cAMP through adenylate cyclase (Mayo, 1992; Gaylinn et al., 1993). cAMP binds to protein kinase A (PKA) resulting in dissociation of the catalytic subunits. From here there are two main pathways

that can be followed. The first results in somatotroph proliferation. PKA catalytic subunits are translocated to the nucleus where they catalyse phosphorylation of transcriptional regulators, such as the cAMP response element binding protein (CREB) (reviewed by Brindle and Montminy, 1992). This leads to gene transcription, for example, of genes containing cAMP response elements (CRE) such as *Pit-1* (Soto et al., 1995) and *c-fos* (Billestrup et al., 1987). The second pathway leads to the secretion of GH. Phosphorylation of PKA leads to subsequent phosphorylation of intracellular and membrane-associated proteins resulting in activation of non-selective cation channels. The subsequent membrane depolarisation stimulates voltage-gated calcium channels leading to an influx of calcium ions. An increase in intracellular calcium triggers exocytosis of the GH secretory vesicles.

These findings have been confirmed with the use of transgenic mouse models. Targeted expression of cholera toxin, an irreversible activator of G_s , to somatotrophs (Burton et al., 1991) resulted in somatotroph proliferation, pituitary hyperplasia and increased serum GH levels. Expression of an inactive mutant of the CREB protein resulted in generation of mice with a dwarf phenotype (Struthers et al., 1991). This phenotype resulted from somatotroph hypoplasia as illustrated by immunofluorescence of transgenic versus non-transgenic pituitaries, indicating that GHRH stimulates cell proliferation as well as secretion. The *little* (*lit/lit*) mouse carries a missense mutation in the GHRH receptor gene (Godfrey et al., 1993; Lin et al., 1993). *Little* mice are unresponsive to treatment with GHRH (Clark and Robinson, 1985b; Jansson et

al., 1986). *In vitro* studies revealed that GHRH can not bind to the mutant receptor (Gaylinn et al., 1999) blocking the stimulation of somatotroph proliferation and GH synthesis and secretion required for normal growth. These dwarf mice have a reduced number of somatotrophs suggesting a proliferative role for GHRH. GHRH receptor mutations have also been diagnosed in humans suffering from isolated growth hormone deficiency (reviewed by Alba et al., 2004). Transgenic overexpression of human GHRH in mice (Hammer et al., 1985; Stefaneanu et al., 1989) has also provided insights into GHRH function. These mice have an increased plasma GH level and accelerated growth and an increased body weight compared to wild type mice. Homozygous GHRH knockout mice are significantly growth retarded with isolated GH deficiency (Alba and Salvatori, 2004). These mice have reduced pituitary GH mRNA and protein content.

1.3.1.2 Somatostatin (SST)

The major inhibitory factor for GH synthesis and secretion is the hypothalamic factor somatostatin (SST). Somatostatin is itself regulated by GH and IGF-1. Hypothalamic SST release increases as plasma GH levels increase (Berelowitz et al., 1982). Infusion of SST in conscious rats led to an inhibition of spontaneous GH release (Clark et al., 1988). Upon removal of SST a large increase in plasma GH was observed (Clark et al., 1988). An increase in plasma GH levels following acute hGH administration leads to inhibition of pulsatile GH secretion in rats (Lanzi and Tannenbaum, 1992). This effect can be blocked by inhibition of SST. SST knockout mice (-/-) maintain normal,

sexually dimorphic growth compared to wildtype littermates. However, the plasma GH profile of males appears feminised and males no longer exhibit a regular pulsatile plasma GH pattern (Low et al., 2001).

It has been suggested that SST inhibits GH secretion by inhibition of cAMP-dependent and -independent calcium ion influx (Reisine et al., 1988). *In vitro* studies using cell culture revealed that SST inhibition of calcium influx could only partially be rescued by administration of pertussis toxin, an inhibitor of inhibitory G proteins (G_i), in turn rendering them unable to inactivate adenylate cyclase. Expression of somatostatin receptor subtypes *in vitro* has shown the ability of SST binding to block cAMP accumulation and therefore signalling (Hou et al., 1994). The G_i proteins, in particular subunit $\alpha i3$, also mediate an increase in inwardly-rectifying potassium channel activity in response to SST (Takano et al., 1997; Chen, 1998). This results in hyperpolarisation of the plasma membrane in turn inhibiting the calcium influx required for GH secretion.

1.3.1.3 Feedback regulation of GHRH and SST

GHRH and SST production is regulated by feedback from GH, as illustrated by a range of studies in the rat. Hypophysectomised rats show an increase in hypothalamic expression of GHRH mRNA and a decrease in SST mRNA expression which can be partially reversed by GH treatment (Chomczynski et al., 1988; Minami et al., 1993). In normal rats with an induced hypersecretion of GH and a subsequently high level of plasma GH, an increase in SST mRNA and a decrease in GHRH mRNA was detected in the hypothalamus (Bertherat

et al., 1993). The dwarf rat (*dw/dw*) has a significantly reduced level of plasma GH due to a reduced number of somatotrophs and decreased pituitary GH content (Charlton et al., 1988; Tierney and Robinson, 2002). This GH deficiency leads to an increase in hypothalamic GHRH expression and the reciprocal decrease in SST expression (Kamegai et al., 1998). A similar pattern of expression is seen in the GH deficient Ames dwarf mouse (Phelps et al., 1993; Hurley et al., 1998). In the transgenic growth-retarded (Tgr) rat, GH deficiency is a result of targeting human GH to GHRH releasing neurons of the hypothalamus (Flavell et al., 1996). Expression of hGH results in local negative feedback to the GHRH neurons resulting in decreased GHRH production and secretion. These rats had reduced GHRH mRNA levels in the hypothalamus.

IGF-1 is also thought to feedback to somatostatin neurons, in turn decreasing GH secretion (Berelowitz et al., 1981). Incubating hypothalamic explants with IGF-1 led to an increase in SST release. This has been illustrated by studies of dwarf rats. An increase in plasma IGF-1 levels following acute administration of IGF-1 led to an increase in hypothalamic SST mRNA and a decrease in GHRH mRNA levels (Sato and Frohman, 1993). Intrapituitary administration of IGF-1 in sheep led to suppression of plasma GH levels (Fletcher et al., 1995).

1.3.1.4 GH Secretagogues

GH secretagogues are a group of synthetic and endogenous peptides that stimulate GH secretion. The first synthetic peptide developed was GHRP-6. This was shown to amplify pulsatile GH release both *in vitro* in cell culture and

in vivo (Bowers et al., 1984). GHRP-6 appeared to act through a novel receptor as no cAMP response was detected upon treatment of primary cultured rat anterior pituitary cells (Cheng et al., 1989), and down-regulation of the protein kinase C pathway blocked the effects of GHRP-6 treatment *in vitro* (Cheng et al., 1991). GHRP-6 treatment of primary cultured anterior pituitary cells resulted in an increase in intracellular calcium, with an increase in radiolabelled inositol phosphates implicating mobilisation of intracellular calcium stores as the major mediator of GHRP-6 induced GH secretion (Lei et al., 1995; Mau et al., 1995).

A unique GH secretagogue receptor (GHS-R) was cloned from human and rat pituitaries (Howard et al., 1996; McKee et al., 1997). In addition to GH cells, GHS-R has also been detected by *in situ* hybridisation in GHRH-expressing neurons of the rat hypothalamus (Tannenbaum et al., 1998). Expression of this receptor was found to be increased in the brain of the dwarf (dw/dw) rat (Bennett et al., 1997) indicating a sensitivity to the impaired GH status of the animal.

An endogenous ligand for the GHS-R was isolated from the rat stomach (Kojima et al., 1999). Termed Ghrelin, this endogenous peptide was shown to be mainly produced by the stomach with some immunopositive neurons found in the arcuate nucleus of the rat hypothalamus (Lu et al., 2001). Ghrelin has been shown to stimulate GH secretion in rats (Seoane et al., 2000). This response is not blocked by treatment with somatostatin (Tolle et al., 2001) suggesting that Ghrelin can inhibit the SST pathway. More recently it has been

shown that an intact GHRH response is required for a GH response to this secretagogue (Tannenbaum et al., 2003). Ghrelin also acts to stimulate feeding behaviour (Wren et al., 2000; Nakazato et al., 2001; Cummings, 2006) and it has been suggested that it may act to integrate the GH axis with energy balance (Tannenbaum et al., 2003).

1.3.1.5 Pattern of GH secretion

The secretion of GH exhibits sexually dimorphic characteristics which are particularly evident in the rat (Tannenbaum and Martin, 1976; Eden, 1979). The peak plasma GH level is normally less in females than in males, except at 45 days when peak plasma GH levels are equivalent. Measurement of plasma GH in conscious rats revealed that male rats have high amplitude, regular peaks of GH secretion (Clark et al., 1986), whereas females have a more variable pattern of secretion, with an increase in pulsatility of secretion observed during the night (Clark et al., 1987).

It has been suggested that this pattern of GH secretion is a result of interactions between GH releasing hormone (GHRH) and somatostatin (SST) in the regulation of GH release. An episodic release of GHRH was recorded in male rats that coincides with a decrease in plasma SST levels (Plotsky and Vale, 1985). Immunoneutralisation of SST led to an increase in basal, trough plasma GH levels in male rats (Painson and Tannenbaum, 1991), while blocking GHRH in this way led to removal of the GH pulses in males and a suppressed response in females.

The pattern of GH secretion has implications in the sexual dimorphism of growth observed in rodents and humans. For example, increased longitudinal bone growth was found when hypophysectomised rats received multiple daily injections of human GH (hGH), compared to those receiving a single daily dose (Jansson et al., 1982). An increased gain in body weight was observed in hypophysectomised rats treated with a pulsatile infusion of hGH compared to continuously dosed animals (Clark et al., 1985). It has also been shown that regular treatment of female rats, and male rats with induced GHRH deficiency, with human GHRH to impose a regular pattern of GH secretion leads to stimulation of weight gain compared to untreated rats (Clark and Robinson, 1985b).

1.3.1.6 Downstream effects of GH

The GH receptor (GHR) is found in many GH target tissues such as liver, muscle and adipose tissue. Binding of GH causes dimerisation of the GH-R by an extracellular interaction (Cunningham et al., 1991). It has been reported recently, however, that dimerisation is not sufficient to activate the GH receptor. Rotation of the receptor subunits relative to one another is thought to result in activation (Brown et al., 2005). Studies of incorporation of ³²-P and immunological assays have been used to show that ligand binding in turn recruits tyrosine kinases (Carter-Su et al., 1989) and phosphorylation of the GH-R (Foster et al., 1988; Campbell et al., 1993). It was later found, by coimmunoprecipitation experiments, that the major tyrosine kinase involved in

GH signalling is JAK-2 (Argetsinger et al., 1993), which associates with a proline-rich motif of the cytoplasmic domain of GH-R (VanderKuur et al., 1994). Phosphorylation of JAK-2 in turn leads to phosphorylation of other cytoplasmic proteins such as Stat (Signal Transducers and Activators of Transcription) 1, 3 and 5 (Campbell et al., 1995; Gronowski et al., 1995; Wood et al., 1995; Smit et al., 1997). The major Stat activated in the liver by GH is Stat5b (Choi and Waxman, 2000). Specific cytoplasmic domains of the GHR must be intact to activate Stat5 phosphorylation (Rowland et al., 2005). This is followed by the rapid translocation of phosphorylated Stats to the cell nucleus (Gronowski et al., 1995) where they initiate transcription of genes responsible for cell proliferation, differentiation and metabolism.

As well as activating the JAK-Stat pathway, binding of GH to its receptor can also activate other signalling pathways. For example, constituents of the Ras-MAP Kinase pathway which leads to the transcription of genes involved in cell proliferation and differentiation have been detected in cultured cell lines in response to GH stimulation (Campbell et al., 1992; Winston and Bertics, 1992). Phosphorylation of Insulin Receptor Substrate (IRS)-1 and 2 (Argetsinger et al., 1995; Argetsinger et al., 1996) has also been studied. These signalling molecules interact with kinases such as PI-3 Kinase which has roles in cell survival, growth and metabolism.

1.3.1.7 Insulin-like Growth Factor (IGF)-I

The main site of IGF-I synthesis is thought to be the liver. Removal of hepatic IGF-I using the Cre//oxP system (Sjogren et al., 2002) resulted in decreased serum IGF-I levels and an increase in serum GH, suggesting a role for IGF-I negative feedback in control of GH secretion. It was also observed that these mice had normal gain in body weight and longitudinal bone growth during puberty. However, further bone development in adulthood was affected compared to normal animals, implying that hepatic IGF-I is required to mediate the growth effects of GH in bone. When a daily treatment of IGF-I was given to Snell dwarf mice, which do not produce GH, an increase in body weight and length, as well as an increase in the size of some organs, was observed (van Buul-Offers et al., 1986). Furthermore, when the IGF-I gene is knocked out by either homologous recombination in embryonic stem cells or generation of a null mutation (Baker et al., 1993; Powell-Braxton et al., 1993) a delay in both embryonic and post-natal bone development is seen. Expression of IGF-I has been detected in many rat tissues as well as the liver (Lund et al., 1986). Non-hepatic IGF-I may have an autocrine/paracrine role.

GH stimulates the secretion of IGF-I into the blood through Stat5b (Davey et al., 2001; Tannenbaum et al., 2001). A Stat5 binding domain has been isolated in the IGF-1 promoter and disruption of Stat5b expression blocks IGF-1 expression *in vivo* in rats (Woelfle et al., 2003a; Woelfle et al., 2003b).

1.3.2 Lactotrophs

Secretion of prolactin from lactotrophs is unusual in that it is predominantly under negative control. The major inhibitory factor is dopamine. Dopamine is synthesised in the cell bodies of the tuberoinfundibular dopaminergic (TIDA) neurons of the arcuate nucleus of the hypothalamus (Kawano and Daikoku, 1987), and is released from the termini of these neurons in the median eminence of the hypothalamus. From here it is carried in the hypophyseal blood to the anterior pituitary where it binds to specific receptors, subtype D2, in the lactotroph plasma membrane to exert its inhibitory effect (Amlaiky and Caron, 1986). Studies of D2 receptor deficient mice have supported the inhibitory role of dopamine (Kelly et al., 1997). These mice have hyperprolactinemia and lactotroph hyperplasia, which results in pituitary adenoma formation with age (Asa et al., 1999).

Secretion of prolactin will occur when the level of dopamine reaching the anterior pituitary falls, lessening the inhibitory effect and enabling other factors such as TSH-Releasing Hormone (TRH) and Vasointestinal Peptide (VIP) to enhance PRL secretion (Martinez de la Escalera et al., 1988). Immunocytochemistry of primary cultured lactotrophs has illustrated the presence of TRH receptors on the plasma membrane of lactotrophs (Yu et al., 1998) and a direct stimulatory role for TRH. VIP, and other members of the secretin family, are present in both the hypothalamus and the pituitary suggesting auto- and paracrine (Gomez and Balsa, 2003), as well as endocrine (Mezey and Kiss, 1985), roles.

Estrogen stimulates lactotrophs to increase PRL synthesis (Shull and Gorski, 1984). Estradiol has also been shown to trigger release of Transforming Growth Factor (TGF) β 3 from lactotrophs, which acts on FS cells to stimulate release of basic Fibroblast Growth Factor (bFGF). This in turn stimulates proliferation of lactotrophs (Hentges et al., 2000; Chaturvedi and Sarkar, 2005). The neuropeptide galanin is thought to have a regulatory role on PRL release and lactotroph proliferation (Kamegai et al., 1998). The amount of galanin expressed in lactotrophs increases in response to estradiol (Kaplan et al., 1988). Female mice with a loss-of-function mutation in the galanin gene fail to respond to estradiol treatment, with no change in cell proliferation or PRL secretion. As a result, these mice have reduced mammary gland development and fail to lactate (Wynick et al., 1998).

The PRL receptor (PRLR) has been isolated in many tissues indicating that PRL has many diverse functions in vertebrates including roles in water and electrolyte balance, lipid and carbohydrate metabolism and growth and development (reviewed by Freeman et al., 2000). However, the major role of this hormone is thought to be in reproduction with the main sites of action for PRL at the mammary gland and ovary. At the mammary gland it stimulates growth and development of the gland during pregnancy, and is essential for milk production including synthesis of the milk proteins casein and lactalbumin (Rillema and Schneider-Kuznia, 1980; Jagoda and Rillema, 1991). Prolactin

also acts to maintain secretion of milk from the mammary gland during lactation (Flint and Gardner, 1994).

PRL is essential for female reproduction as shown by studies of PRL and PRLR knockout mice. PRL knockout females are completely infertile with irregular oestrus cycles (Horseman et al., 1997). They exhibit abnormal development of the mammary gland in adult females, with a failure to form the lobular structures associated with normal adult mammary glands. Mice carrying a null mutation in the PRLR gene (PRLR knockout) (Ormandy et al., 1997) have similar characteristics to the PRL knockout mouse. Heterozygous female mice had a reduction in the level of mammary gland development, which led to an inability of these mice to lactate with their first litter. Subsequent litters increase the lactational ability of these mice. Homozygous female mice are sterile and have an altered estrous cycle. Oocyte fertilisation did occur but very few blastocysts were recovered from these females indicating that embryonic development is terminated shortly after fertilisation. It is suggested that this is due to an inability of the corpus luteum to respond to PRL and hence maintain estrogen and progesterone levels necessary for embryo survival and implantation.

PRL binding to the PRL receptor results in activation of the receptor complex and association of the tyrosine kinase JAK2, as previously described for GH signalling (Freeman et al., 2000). *In vitro* studies of a PRL-dependent cell line, Nb2, have revealed the possibility that other signalling pathways may also be involved. For example, the MAPK signalling cascade has been identified as a

pathway in PRL induced cell proliferation (Clevenger et al., 1994). The mammalian TOR (mTOR) pathway, involved in initiation of translation and cell growth, has also been implicated in PRL signalling (Boudreau et al., 2002). Proliferation of Nb2 cells was reduced in the presence of rapamycin, which binds to intracellular binding proteins such as FKBP to inhibit association of components of the mTOR pathway.

1.3.2.1 Lactotroph plasticity

The lactotroph cell population shows a high degree of plasticity. This is most pronounced in females where a large increase in cell number occurs to accommodate the increase in prolactin that is required during pregnancy and the lactation period that follows. During pregnancy the pituitary gland increases in size, with the largest glands seen immediately postpartum (Elster et al., 1991; Dinc et al., 1998). After weaning, the amount of prolactin required is reduced and the number of lactotrophs decreases to its pre-pregnancy level (Haggi et al., 1986). The exact mechanism for how this increase and decrease occurs is unknown, but several theories have been suggested:

- that it occurs by proliferation of the current lactotroph population (Stefaneanu et al., 1992) followed by apoptosis; or,
- that transdifferentiation occurs from the somatotroph to the lactotroph (Stefaneanu et al., 1992) followed by a reverse transdifferentiation step back to the somatotroph (Vidal et al., 2001); or,

- that differentiation of the Pit-1-dependent precursor cell is driven towards lactotroph production followed by a decrease in differentiation and an increase in apoptosis; or,
- that it is a combination of the above processes.

For transdifferentiation to occur it has been suggested that there is an intermediate cell type that contains both growth hormone and prolactin, for example the mammosomatotrophs.

1.3.3 Mammosomatotrophs

Mammosomatotrophs are cells of the anterior pituitary that have the ability to synthesise and secrete both GH and PRL. These cells were described by Frawley and colleagues (Frawley et al., 1985) as a subpopulation of primary cultured rat anterior pituitary cells that could simultaneously secrete GH and PRL, when studied by sequential haemolytic plaque assay. Subsequent studies confirmed this observation (Leong et al., 1985). Immunogold electron microscopy revealed the presence of these cells *ex vivo* in male and cycling and lactating female rat pituitaries (Nikitovitch-Winer et al., 1987) and their development in the postnatal rat pituitary (Huerta-Ocampo et al., 2005). These observations led to the suggestion that mammosomatotrophs could be either a precursor cell for somatotrophs and lactotrophs (Nikitovitch-Winer et al., 1987; Borrelli et al., 1989), or an intermediate cell type, allowing the interconversion between somatotrophs and lactotrophs (Porter et al., 1990; Porter et al., 1991;

Vidal et al., 2001), for example during pregnancy and lactation when lactotroph cell number increases.

1.3.4 Thyrotrophs

Synthesis and secretion of TSH from thyrotrophs is mainly controlled by TRH and thyroid hormone (TH). TRH is released from the hypothalamus and stimulates secretion of TSH from the pituitary (Harris et al., 1978a; Steinfelder et al., 1991). Studies of the TRH knockout mouse (Yamada et al., 1997) indicate that the major role for TRH is thyrotroph secretion and not regulation of TSH synthesis as thyrotroph development can still occur in the absence of TRH (Nikrodhanond et al., 2006).

TH is released from the thyroid in response to TSH (reviewed by Kohrle, 1990). High levels of TH negatively feed back to the pituitary to down regulate TSH gene expression and secretion. Upon removal of this feedback, for example following thyroidectomy, the number (or proportion of) thyrotrophs in the pituitary increases (Nolan et al., 2004). An increase in the plasma levels of TSH and TH has been reported in TH receptor- β knock out mice (Shibusawa et al., 2003; Nikrodhanond et al., 2006). TH specific receptors have been identified in folliculostellate cells as well as thyrotrophs suggesting indirect modulation may also play a role in thyrotroph regulation (Alkemade et al., 2006). Other factors are thought to play a role in TSH regulation, for example neuromedin B (Oliveira et al., 2006).

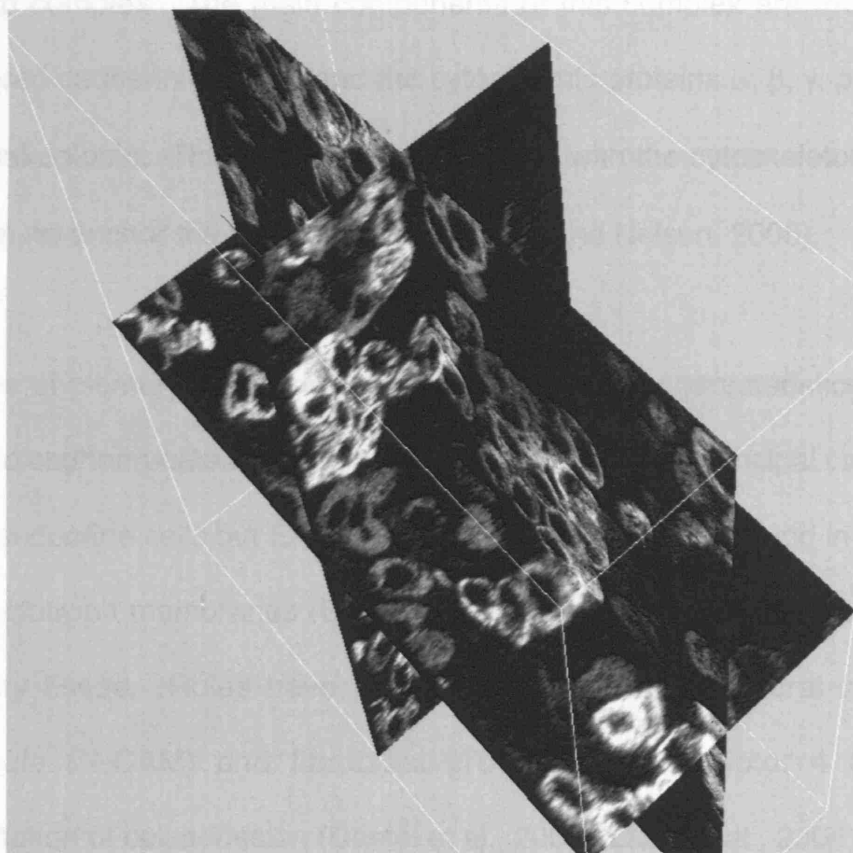
1.4 Cell networks and connections within the anterior pituitary

Previous work by Fauquier *et al.*, 2001, has shown that folliculostellate (FS) cells form a communicative network that surrounds the endocrine cells of the anterior pituitary. Calcium recording and imaging studies revealed the ability of these cells to carry signals over a long distance. This is thought to be due to the existence of gap junctions between the FS cells, as shown by blocking of measurable activity in the presence of carbenoxolone.

It has recently been shown that the somatotroph population forms a continuous network throughout the anterior pituitary (Bonnetfont *et al.*, 2005). Using 2-photon microscopy of GH-eGFP mouse (Magoulas *et al.*, 2000) pituitaries, it was demonstrated that in adult male mice all GH cells are in contact with another GH cell (figure 1.2). This network consists of strings of cells and cell clusters that develops from e15.5 and persists to old age. These imaging studies have revealed plasticity of the somatotroph network with age. For example, at 60 days of age male mice have a greater number of large clusters in the lateral zone of the anterior pituitary than the median zone. At both prepubertal and adult (100 days) ages the proportion of strands and clusters is similar between the two zones indicating an ability to adapt to requirements for GH. The formation of large clusters is a male-specific phenomenon. Imaging analysis of female and castrated male pituitaries showed a decrease in cell clustering in line with the difference in body weight. Immunohistochemistry for β -catenin revealed that adherens junctions maintain the GH cell network.

A second mouse has been developed in the lab that expresses the fluorescent tag DsRed in the cytoplasm of lactotrophs (Fernandez-Fuente and Le Tissier, unpublished). Imaging of the Prl-DsRed mouse pituitary indicates that PRL cells may also form a network within the anterior pituitary, although the structure of this is thought to differ from that of GH (Fernandez-Fuente, Robinson, Lafont, Mollard, Le Tissier – personal communication). The contacts that form between PRL cells are different to those observed in the GH cell network and it has been suggested that contact may be maintained by gap junctions.

Figure 1.2 3D orthogonal slicing of a 2-photon image stack of a GH-eGFP male pituitary (Bonnefont et al., 2005).



1.4.1 Gap junctions

Gap junctions are found at points of close proximity (2-4nm) of the plasma membrane of neighbouring cells as illustrated by electron microscopy of smooth muscle (Uehara and Burnstock, 1970). The junction is formed by pairing of connexins between neighbouring cell membranes. The main role of these junctions is to allow communication between cells by movement of small molecules (<1KDa in mammalian cells) (Flagg-Newton et al., 1979).

1.4.2 Adherens junctions

Adherens junctions maintain cell-cell interactions by the formation of a cadherin-catenin complex. The main components of this complex are the membrane-anchored cadherin (E/N/R), and the cytoplasmic proteins α , β , γ , p120 catenins and plakoglobin. This complex then interacts with the cytoskeleton, through α -catenin, to anchor the cell (reviewed by Weis and Nelson, 2006).

Studies of pituitary adenomas have revealed many characteristics of pituitary-specific cadherin-catenin complexes. N-cadherin is the principal cadherin of the neuroendocrine cells but E-cadherin is the major cadherin found in somatotroph and lactotroph membranes (Ezzat et al., 2006a) in adenomous but not normal pituitary tissue. Roles have also been suggested for neural cell adhesion molecule (N-CAM) and fibroblast growth factor receptor 4 (FGFR-4) in stabilisation of cell adhesion (Daniel et al., 2000; Ezzat et al., 2004).

The first half of this introduction describes the physiology of the pituitary gland, with particular focus on cells of the Pit-1 lineage. The overall aim of my thesis is to study the interactions of the somatotroph and lactotroph populations and the possibility for interconversion between the two. In order to achieve this I will be using a transgenic mouse model. Consideration must be given to the promoter and the possible methods that could be used to achieve conditional, temporal control of the transgene. This is taken into account in the following sections.

1.5 Somatotroph-targeted transgene expression

When creating a transgenic model it is important to consider the type of promoter to be used to drive specific expression of the transgene *in vivo*. Two examples of promoter that have been used to direct transgene expression to the somatotroph are a short hGH proximal 5' promoter sequence and the large hGH locus control region (LCR).

Early transgenic models made use of a short promoter sequence to specify somatotroph expression. For example, Behringer *et al.*, 1988, used a 310bp fragment of the rat GH promoter fused to a fragment of the hGH gene containing the promoter (-83bp upstream) and part of the first exon. Borrelli *et al.*, 1989, used only a 320bp fragment of the rGH promoter to drive expression of thymidine kinase. These promoters contain enhancer elements to drive pituitary specific expression (Nelson *et al.*, 1986). However, the resulting

founders showed a range of transgene expression, requiring selective screening for breeding.

In order to improve the consistency and level of expression of somatotroph-targeted genes *in vivo* the hGH locus control region (LCR) can be used (Jones et al., 1995; Su et al., 2000). This human-specific LCR has been shown to give a copy number-dependent, integration-independent pattern of expression of transgenes in the mouse (Magoulas et al., 2000). Such a region of genetic control is not present in the rodent GH gene. The hGH LCR is located -14.5kb to -32kb distally of the hGH gene cluster and is marked by five DNase I hypersensitive sites (HS I-V). HS I and II (-14.5kb – -16.2kb upstream of the coding sequence (Bennani-Baiti et al., 1998)) contain all of the Pit-1 binding sites required for acetylation during transcription of pituitary GH (Shewchuk et al., 1999; Kimura et al., 2004) that is necessary for long-range activation of the gene (Ho et al., 2006). HS III-V are required for placental expression of hGH-V (hGH variant) from the hGH gene cluster (Jones et al., 1995; Elefant et al., 2000). The hGH LCR has been used to successfully target expression of eGFP to somatotrophs (Magoulas et al., 2000). Integrity of the hGH LCR was maintained resulting in a high level of somatotroph-specific eGFP expression. I have used the hGH LCR in all of my studies.

1.6 Fluorescent labelling of the somatotroph population

The use of fluorescent-tags is a widely exploited tool for labelling specific cell types to aid imaging studies of cellular and subcellular characteristics.

Enhanced green fluorescent protein (eGFP) is a modified form of GFP that has an improved fluorescence intensity and higher expression levels in mammalian cells both *in vitro* and *in vivo* (Yang et al., 1996). eGFP has been targeted to somatotrophs *in vivo* (Magoulas et al., 2000) in order to study the secretory processes in single cells and at the subcellular level. The hGH LCR was used to ensure somatotroph-specific expression. The first 48 amino acids of the hGH gene product was included upstream of eGFP. This amino acid sequence contains the signal peptide and N-terminal 22 residues of hGH. Analysis of fluorescence *in vitro* and *in vivo* by immunohistochemistry revealed colocalisation of endogenous GH and GH-GFP in a punctate distribution, suggesting successful targeting to secretory vesicles. This was confirmed by double immunogold electron microscopy (Magoulas et al., 2000).

1.7. Genetic manipulation methods to alter the function of a specific cell population

Several methods have been employed by groups to specifically alter the function of a cell population *in vivo* in a conditional and/or temporal manner and study the effects of this manipulation in a physiological context. Below is an outline of these generic techniques, and where relevant their application to the somatotroph population.

1.7.1 Thymidine kinase

Thymidine kinase is an enzyme synthesised by the Herpes Simplex Virus. This protein can convert nucleoside analogs, such as the drugs FIAU (1-(2-deoxy-2-

fluoro- β - δ -arabinofuranosyl)-5-iodouracil) and gancyclovir designed to specifically target the viral thymidine kinase (Mansuri et al., 1987), into toxic intermediates. These intermediate products disrupt DNA replication of the cell resulting in rapid cell death (Borrelli et al., 1988).

1.7.1.1 Dwarfism resulting from expression of thymidine kinase

Herpes Virus 1 thymidine kinase (HSV1-TK) has been targeted to several pituitary cell lineages to ablate cell populations and study downstream effects in mouse models. The first transgenic model designed using this method was one of inducible dwarfism (Borrelli et al., 1989). Here, HSV1-TK was targeted to somatotrophs. Upon treatment with the drug FIAU at p2 transgenic mice developed as dwarfs. Immunocytochemistry of the pituitaries of these mice revealed an almost complete ablation of the somatotroph and lactotroph populations when mice were treated from this early age. When the drug was removed, and animals allowed to recover from the treatment, an increase in somatotroph number and a small increase in lactotroph number was observed. This model provided evidence for the developmental relationship between somatotrophs and lactotrophs. This technique has also been applied to the studies of corticotroph (Allen et al., 1995) and gonadotroph (Markkula et al., 1995) development and effects on their downstream targets.

The main disadvantage of this model is that cell death will only occur in the presence of the drug in actively dividing cells. For example, in the model of inducible dwarfism (Borrelli et al., 1989), mice that were not treated with the

drug until p10 grew to a normal size and had a greater number of somatotrophs remaining. A second problem with this method is that bystander cell death has been reported, both *in vitro* and *in vivo*, following treatment with gancyclovir (Freeman et al., 1993; Mesnil et al., 1996). Cells that do not express the thymidine kinase transgene but are in close proximity, or connected, to thymidine kinase positive cells can also be ablated in the presence of the drug. It is not clear how this occurs although two possible explanations are that negative, neighbouring cells engulf the apoptotic vesicles released from the dying (positive) cells (Freeman et al., 1993), or that the toxic effect is transmitted to neighbouring (negative) cells through gap junctions (Mesnil et al., 1996).

1.7.2 Diphtheria toxin A

Diphtheria toxin subunit A (DT-A) is a cytoplasmic enzyme produced by the Diphtheria bacterium. It inhibits cellular protein synthesis by catalysing the addition of ADP-ribose from NAD to a specific modified histidine residue, known as diphthamide, in Elongation Factor 2 during translation (reviewed by Collier, 1975). It is thought that only one molecule of DT-A is required to kill a cell (Yamaizumi et al., 1978) making it a highly efficient method for inducing cell death.

This method of cell ablation has been specifically targeted to several mouse tissues including the pancreas (Palmiter et al., 1987) and the lens of the eye (Breitman et al., 1989; Kaur et al., 1989). However, expression of the highly

toxic wildtype DT-A can have adverse side effects such as embryonic lethality and bystander cell death. A second, mutated form of DT-A was isolated (Maxwell et al., 1987). This protein has a reduced toxicity compared to the wildtype form, which may prove beneficial when generating transgenic mouse lines expressing DT-A.

1.7.2.1 Somatotroph ablation with Diphtheria toxin transgene

DT-A has been used to target cell specific cell ablation to somatotrophs (Behringer et al., 1988). Here, DT-A was inserted into the first exon of rat GH and targeted to somatotrophs using 310bp of the rGH 5' flanking sequence. The resulting founders were dwarf and subfertile. Lines could not be maintained through transgenic females and *in vitro* fertilisation was required to maintain the male line. Serum GH was undetectable in transgenic mice by RIA and IGF-1 levels were significantly reduced. Transgenic mice showed anterior pituitary hypoplasia and immunohistochemistry for GH revealed very few somatotrophs remained. Somatotroph ablation is irreversible, however, using this model. A reduction in lactotrophs was also apparent although to a lesser extent than somatotrophs. From this they hypothesised that PRL cells must be able to differentiate from a precursor cell independent of GH, in order for lactotrophs to survive somatotroph ablation.

1.7.3 Diphtheria toxin receptor

The diphtheria toxin receptor (DTR) has been targeted to hepatocytes to provide an inducible model of cell ablation (Saito et al., 2001). The DTR is a

precursor, membrane-anchored form of the heparin-binding EGF-like growth factor (HB-EGF). HB-EGF is found in many species. The human precursor form has been shown to bind diphtheria toxin (Mitamura et al., 1995), whereas those in mice and rats do not. In this transgenic model, Saito *et al.* (2001) targeted the human form of the HB-EGF precursor to mouse cells, using a hepatocyte-specific promoter. Administration of low doses of diphtheria toxin to these mice resulted in induction of hepatocyte ablation.

1.7.4 Cre recombinase

The bacteriophage Cre/*loxP* system (Sternberg and Hamilton, 1981) has been exploited for *in vivo* use. Cre recombinase recognises specific DNA sequences termed *loxP*. It will mediate excision of the DNA fragment contained within two *loxP* sites when expressed with the floxed DNA (*loxP*-DNA-*loxP*). The *loxP* sites can be inserted around the coding sequence for a specific gene or a sequence encoding a transcription stop site. In order to use this system *in vivo* two transgenic lines must be generated. The first line expresses Cre recombinase targeted to a specific cell lineage. This line is then crossed to a second line containing the floxed sequence. For example, a ubiquitously expressed promoter such as Rosa26 (Soriano, 1999) can be used to drive expression of a reporter such as *lacZ* or a fluorescent protein. Upstream of the reporter sequence is a DNA fragment that will halt transcription/translation before the reporter sequence is reached. When the Cre and reporter lines are crossed, the stop sequence is excised from DNA and transcription of the

reporter will occur. This will then mark any cell that expresses, or has expressed, both transgenes.

1.7.4.1 GH-Cre

Cre recombinase has been used to mark the cells of the somatotroph lineage (Luque et al., 2007). A short rat GH promoter (310bp), previously shown to give a high level of somatotroph-specific expression (Behringer et al., 1988), was used to target Cre recombinase expression to somatotrophs. This mouse line was crossed to a second, double reporter, mouse line with ubiquitously expressed floxed LacZ/human placental alkaline phosphatase (Z/AP (Lobe et al., 1999)). Upon recombination, the floxed LacZ sequence is removed allowing expression of the human placental alkaline phosphatase (hPLAP) sequence. This technique allows all cells that express, or have once expressed, GH to be marked. hPLAP expression was detected in the anterior pituitary and testes of double positive mice. Immunocolocalisation of hPLAP and anterior pituitary hormones was used to test the specificity of the rGH-Cre model. No colocalisation was found with hPLAP and ACTH, LH or TSH. A small percentage of lactotrophs were positive for hPLAP but it was not determined if these were mammosomatotrophs. It was concluded that this may indicate that a small subpopulation of lactotrophs differentiate from a GH-expressing cell. Alternatively, if these cells are positive for GH and PRL (not assessed in this model), it may indicate that somatotrophs differentiate from a double positive precursor cell.

Similar Cre recombinase techniques have been used for fate mapping of other specific cell lineages, for example during embryonic development (Zinyk et al., 1998; Jiang et al., 2000; Luque et al., 2007). More recently it has been developed to target ablation strategies to a cell lineage (as described below).

1.7.4.2 Cre-DTR

In this model Cre recombinase has been used to induce expression of the diphtheria toxin receptor (iDTR) in T- and B- cells (Buch et al., 2005). Cre-iDTR mice are unaffected by expression of the receptor. However, upon injection of diphtheria toxin cell ablation was induced. This resulted in a decrease of T- and B- cells and efficiency of ablation could be improved by prolonged injection of the toxin.

1.7.4.3 Cre-DT-A

In a second approach DT-A was targeted to cells expressing Nkx2.5 by Cre recombination in this specific cell lineage (Ivanova et al., 2005). In this model Cre recombinase was driven by either the Nkx2.5 (heart) or Wnt1 (midbrain) promoter and DTA expression was driven by Rosa26, containing a floxed eGFP-*neo* cassette. In the absence of Cre all Rosa26 expressing cells will express eGFP but DT-A expression is blocked by the presence of the neomycin cassette. Cre recombination removes the eGFP-*neo* sequence and allows DT-A expression inducing cell ablation.

1.7.4.4 Inducible Cre recombination

Cre recombination provides a model for spatial control of expression of a floxed transgene. One disadvantage of this is that the point in development at which Cre recombination occurs is dependent on promoter activity of the transgene construct. One way to overcome this is to create a model of inducible recombination. Several methods have been designed to achieve this, for example, tamoxifen inducible Cre. Here Cre recombinase is fused to the hormone binding domain of a mutated, tamoxifen-responsive estrogen receptor (Cre-ER^T). This fusion protein is retained in the cytoplasm in the absence of Tamoxifen. Upon treatment with the drug Cre-ER^T is translocated to the nucleus resulting in recombination of the floxed DNA (Metzger et al., 1995; Feil et al., 1996). This method has been used to induce Cre recombination postnatally at a range of sites in the mouse, including endothelial cells (Forde et al., 2002), kidney (Dworniczak et al., 2007), and various regions of the central nervous system (Doerflinger et al., 2003; Hirrlinger et al., 2006; Bradley et al., 2007). Other methods have also been developed. For example, in one model, inducibility was achieved using a promoter containing an hypoxia-responsive element (HRE) and a radiation-responsive element (CArG) (Greco et al., 2006). Cre recombination could be induced by hypoxia and/or radiation treatments to drive thymidine kinase expression in mice with developed carcinomas, which resulted in cell ablation upon treatment with gancyclovir.

1.7.4.6 FLP recombinase

An alternative to Cre recombinase is the yeast recombinase FLP. This too recognises specific recombinase target sites in the DNA, known as FRT (FLP-recombinase target) sites. Use of FLP recombinase has also been used as a method to conditionally alter a specific cell type *in vivo*. For example, FLP recombination has been targeted to the Wnt1 cell lineage in mice (Dymecki, 1996). A mouse line transgenic for *Wnt1-FLP* was crossed to a line carrying a ubiquitously expressed FRT-LacZ. Upon recombination LacZ was expressed in all Wnt1 expressing cells, as detected by β -galactosidase activity. Activity of this recombinase can also be induced by combining with the ER^T discussed above, providing another level of control (Hunter et al., 2005).

1.7.5 Tet-On/Off

The tet-system provides a model for reversible induction of a transgene by addition or removal of a tetracycline antibiotic, usually doxycycline. Fusion of the bacterial, DNA binding Tet repressor with the transcriptional activation domain of virion protein 16 of herpes simplex virus produces the transcriptional transactivator (tTA) that is the basis for the Tet-Off system, where DNA binding of tTA is inhibited by tetracycline (Gossen and Bujard, 1992). Replacement of the original Tet repressor with a mutant form from *E. coli* generates a second model, known as rtTA or Tet-On, which enables tetracycline-induction of gene expression (Gossen et al., 1995). In both cases removal of the drug reverses the effect induced by drug treatment.

This system has been targeted to several sites *in vivo*. In order to generate a transgenic line responsive to tetracycline an initial cross of two mouse lines must be carried out. Only double transgenic mice will be responsive to the drug treatment. In early transgenic experiments (Furth et al., 1994), the first transgenic line used expressed tTA from the human cytomegalovirus (hCMV) immediate early gene 1 promoter. This was crossed to a second line expressing either a luciferase or β -galactosidase reporter gene from the minimal promoter of hCMV. Transcription of the transgene is suppressed in the presence of tetracycline but upon removal of the drug a several thousand-fold increase in expression was observed. Refinement of this model has led to cell specific targeting of Tet expression. For example, transgenic mouse lines targeting reporter expression to tissues such as the mammary gland (Gunther et al., 2002) and heart (McCloskey et al., 2005) have been generated.

Although the Tet model can provide temporal control *in vivo* there are several problems associated with it. For example, unless it is intended to study a cell lineage during embryonic development with the Tet-Off system, it is necessary to treat pregnant females with tetracycline. Expression of toxic transgenes can result in embryonic lethality in the absence of the drug (Lee et al., 1998). It should also be noted that this in turn has associated problems as doxycycline can affect placental function and foetal development (Moutier et al., 2003). Some background leakiness of the Tet system has been reported (Gossen and Bujard, 1992; Gossen et al., 1995) reducing cell specificity of the model.

More recently, improvements to the Tet system have enabled it to be used to temporally deliver cytotoxic substances to a specific cell lineage to alter function at a given point in development, as outlined below.

1.7.5.1 Tet-DTA

As previously described DT-A expression can result in cell specific ablation. To gain temporal control of the initiation of cell ablation a Tet-Off strategy has been employed (Lee et al., 1998). In this transgenic model DT-A was targeted to adult mouse heart to mimic effects seen in cardiomyopathies with tetracycline repressing DT-A transcription. Upon removal of the drug DT-A expression can occur resulting in cell ablation and onset of several of the symptoms associated with the human disorder. In a second model, expression of the mutated form of DT-A in a subpopulation of olfactory sensory neurons using rtTA resulted in temporal control of DT-A transcription and cell death (Gogos et al., 2000). Terminating the drug treatment prevented DT-A expression and enabled regeneration of the neuron subpopulation.

1.7.5.2 Tet-barnase

In this model, induction of a suicide gene, *barnase*, causes cell ablation upon removal of tetracycline (Leuchtenberger et al., 2001). *Barnase* is a RNase from the bacterium *Bacillus amyloliquefaciens* (reviewed by Hartley, 1989). Induction of barnase following tetracycline removal is irreversible in the targeted cells. However, by reinitiating tetracycline treatment it may be possible to study

regeneration of the targeted cell population, assuming the presence of an unaffected progenitor cell population.

1.7.5.3 Tet-controlled Cre recombinase

Tetracycline can also be used to control Cre recombination. This utilises an expression vector encoding Cre as well as the tetracycline operator and repressor. Upon treatment with tetracycline, the repressor element is released from the operator allowing transcription of Cre recombinase and subsequent recombination of the target floxed gene to occur. This method has been used to target temporally regulated Cre recombination to pituitary gonadotrophs (Naik et al., 2006).

1.7.6 Inducible transgene expression using a cytochrome p450 promoter

Cytochrome p450 1a1 (Cyp1a1) is found in several mouse tissues including the liver, lung, kidney and skin. Its expression is inducible on exposure to aryl hydrocarbons such as indole-3-carbinol (I3C). This is mediated by the transcription factor aryl hydrocarbon receptor which binds to specific elements in the Cyp1a1 promoter. This promoter has been used to drive I3C-inducible expression of transgenes in mouse and rat (Campbell et al., 1996; Kantachuvesiri et al., 2001). Expression of the transgene only occurs in the presence of I3C. Upon removal of the drug, the phenotype is reversed. For example, induction of the transgene Cyp1a1-Ren2 causes hypertension in the rat (Kantachuvesiri et al., 2001). This phenotype is normalised when I3C is removed from the diet.

1.7.7 Destabilisation of an endogenous protein to induce a phenotype

It is also possible to conditionally alter the function of a specific cell type by expression of unstable small molecules such as FRB*. FRB* (FKBP/rapamycin binding) protein domain is thermally unstable. Fusion of a target protein with this tag will confer instability to this protein leading to its rapid degradation. Treatment with rapamycin stabilises the FRB* tag and in turn the protein, rescuing the induced phenotype. This has been applied to the study of midline defects occurring during mouse development by tagging GSK3 β , a protein with regulatory roles throughout development (Liu et al., 2007). In the absence of rapamycin GSK3 β -FRB* mice are phenotypically identical to the GSK3 β null mutant, with cleft palate and sternal defects. Treatment with rapamycin prevents the occurrence of these defects.

1.7.8 A transgenic mouse model with autosomal dominant GH deficiency

Isolated GH deficiency II (IGHD II) is an human inherited autosomal dominant form of IGHD that is associated with a splicing event, producing an exon 3 skip product of GH that is 17.5 kDa in size (Cogan et al., 1994; Binder and Ranke, 1995). This dominant negative form of GH disrupts wildtype hGH production and secretion (Hayashi et al., 1999). The result is variably decreased plasma GH levels and growth rates.

In order to study the mechanism for disruption of somatotroph function, the exon3 skip product (Δ_{exon3} hGH) was targeted to somatotrophs *in vitro* and *in vivo* using the hGH LCR to drive expression and GFP as a cellular marker

(McGuinness et al., 2003). *In vitro* studies of cultured cell lines indicated that expression of the Δexon3 hGH construct caused increased cell death with a greater number of detached, shrivelled cells and cell debris visible in transfected cultures, with the remaining cells containing very few secretory vesicles. No exocytotic events were detected using TIRF microscopy of GFP-tagged Δexon3 hGH transfected cells. Generation of transgenic mice using this construct yielded 3 lines, two of which contained a high copy number of the transgene and showed significant dwarfism from 3 weeks of age compared to non-transgenic littermates. Both male and female transgenic mice of these lines were GH, PRL, TSH and LH deficient with marked anterior pituitary hypoplasia. The third line contained a lower copy number of the transgene, showed relatively normal growth and had late onset GH deficiency with a slight reduction in pituitary PRL as measured by RIA. Very few somatotrophs could be identified in the transgenic lines by electron microscopy. Those imaged had enlarged ER, golgi and mitochondria with few, irregular shaped secretory vesicles. Numerous macrophages were identified through the anterior pituitary of the higher copy number lines, which may have contributed to the decreased number of lactotrophs, corticotrophs and gonadotrophs imaged. McGuinness *et al.* (2003) hypothesised that the lower copy line was the most representative of human IGHD II with the hormone deficiency confined mainly to GH, though later studies in humans with IGHD II has shown that other hormone axes are also affected (Mullis et al., 2005; Salemi et al., 2005).

1.7.9 Immunoablation of a cell population

To study the consequences of antigen recognition in the anterior pituitary, an antigen, influenza nucleoprotein, was expressed in somatotrophs of Rag1 ^{-/-} mice using the hGH LCR (de Jersey et al., 2002). Rag1 ^{-/-} mice have no B or T cells. The influenza nucleoprotein was stored in somatotroph secretory vesicles and released with GH to create high local concentrations of antigen. When these mice were crossed to F5 TCR (T Cell Receptor) transgenic mice, which express a monoclonal population of CD8 T cells specific for influenza nucleoprotein, a dramatic reduction in the pituitary GH content was observed with age. This was attributable to endocrine cell apoptosis and infiltration of CD8 T cells into the anterior pituitary, as observed with immunostaining. These double transgenic mice were dwarf compared to control littermates. PRL and TSH deficiencies also developed with age.

1.7.10 Conditional silencing of a cell population

The glutamate-gated chloride channels are a family of inhibitory ion channels specific to invertebrate organisms (Cully et al., 1994; Cully et al., 1996; Forrester et al., 1999; Tandon et al., 2006). *In situ*, the channel opens in response to the binding of glutamate resulting in hyperpolarisation of the cell membrane leading to inhibition of the nerve impulse. These channels are the target of the anthelmintic drugs, such as Ivermectin, which paralyse the feeding muscle of the parasitic worm. These drugs have a low toxicity to the host animal at the doses required to kill the worm (Campbell et al., 1983; Sutherland

and Campbell, 1990). This discovery makes it a possibility for this channel to be used as a conditional silencer *in vivo*.

Codon optimisation, modification and fluorescent labelling of the α and β subunits of the glutamate-gated chloride channel have enabled *in vitro* studies (Slimko et al., 2002) and preliminary *in vivo* work (Lerchner et al., 2007) to be carried out to show that reversible silencing can be achieved in neurons with Ivermectin. However, *in vivo* work has revealed difficulties with targeting the channel to a specific cell type (Slimko et al., 2004).

1.7.11 Using the M2 ion channel for conditional ablation of a specific cell type

M2 is a homotetramer, where each subunit contains a single transmembrane domain. The M2 protein of the Influenza A virus forms an active ion channel, as shown by expression in *Xenopus laevis* oocytes (Pinto et al., 1992). It is essential for acidifying the interior of infecting virions during uncoating following endocytosis (Bukrinskaya et al., 1980; Bukrinskaya et al., 1982). Native M2 is gated by pH (Chizhnikov et al., 1996). When the external pH is low (high proton concentration) a conformational change occurs at a single tryptophan residue in the transmembrane domain of the pore, opening the channel and allowing translocation of protons into the virion (Tang et al., 2002). A second residue, Histidine 37, is necessary for ion selectivity and activation by the low external pH (Wang et al., 1995).

The adamantane drug group (Amantadine, Rimantadine and their derivatives) bind in the channel pore preventing proton translocation (Lin et al., 1997). These drugs were designed to complement the shape, hydrophobicity and polarity of the pore to displace the water molecules required for proton movement (Salom et al., 2000). Blocking the M2 channel impairs virus uncoating by preventing the dissociation of M1 (matrix protein) and the RNP (ribonucleoprotein) core that occurs at low pH (Bukrinskaya et al., 1982). It also thought to have a secondary effect on expression and conformation of haemagglutinin, a protein necessary for endo and exocytosis of the virus (Daniels et al., 1985).

Mutation of the transmembrane amino acid Histidine 37 to alanine removes the proton specificity of the channel and broadens specificity to other monovalent cations (Na^+ and K^+) (Smith et al., 2002). This mutant channel is 'open' at physiological pH. Sensitivity to Rimantadine remains despite this mutation (D. Ogden, unpublished). H37A M2 has been targeted to T cells (Smith et al., 2002) and GHRH neurons (Le Tissier et al., 2005) in an attempt to produce transgenic mouse models of reversible ablation of specific cell populations. Treatment of these models with amantadine failed to reverse cell ablation *in vivo*. However, reversal of T cell ablation was achieved following *in vitro* culture of transgenic foetal thymic lobes, containing progenitor T cells, in the presence of amantadine suggesting that the inability to reverse the phenotype postnatally *in vivo*, and in adult thymic lobes *in vitro*, is a result of complete ablation of the progenitor population or access of the drug *in vivo*. More recently, targeting of

H37AM2 to heart and macrophage cells in *Xenopus laevis* embryos (Smith et al., 2007) suggests that M2 is required to accumulate before cell ablation, possibly through apoptosis, is induced. Reversibility was achieved by bathing tadpoles in a Rimantadine solution.

1.8 Aims

The overall aim of my thesis is to study interactions of the somatotroph and lactotroph populations and their proliferation and differentiation under different physiological conditions. I decided to try to do this using a transgenic mouse model expressing the viral ion channel M2 to achieve somatotroph ablation controllable with Rimantadine. Conditional, temporal ablation of the somatotroph population will enable the study of the dependence of the lactotroph population upon somatotrophs during pituitary development and during periods of lactotroph plasticity, for example during pregnancy, lactation and weaning. The hope was that the presence of a pool of adult progenitor cells within the pituitary would allow renewal of the somatotroph population when M2 activity is blocked *in vivo* with Rimantadine treatment. It might also offer the possibility of studying functional control of somatotroph excitability and release with the use of ion channel blockers in the presence and absence of Rimantadine.

Preliminary work by Sarah Lilley, a previous post-doc in Neurophysiology, was carried out to test the effect of M2 channel expression on GH cell function *in vitro* (Le Tissier et al., 2005), as it was unclear whether these cells would be

affected by channel expression in the same way as neurons. GC cells were transiently transfected with the transgene and whole-cell voltage-clamp assays used to depolarise the cell and test the activity of the ^{H37A}M2 channel in the presence and absence of Rimantadine (figure 1.3). M2 is expressed in a constitutively active form and requires the presence of the drug to block activity. In the presence of Rimantadine, channel activity is suppressed. Activity returns to pre-treatment levels after removal of the drug, indicating reversibility of the system *in vitro*.

The hGH LCR has been used to target ^{H37A}M2 expression to GH cells. For simplicity the transgene will be referred to as GH-M2 from here on.

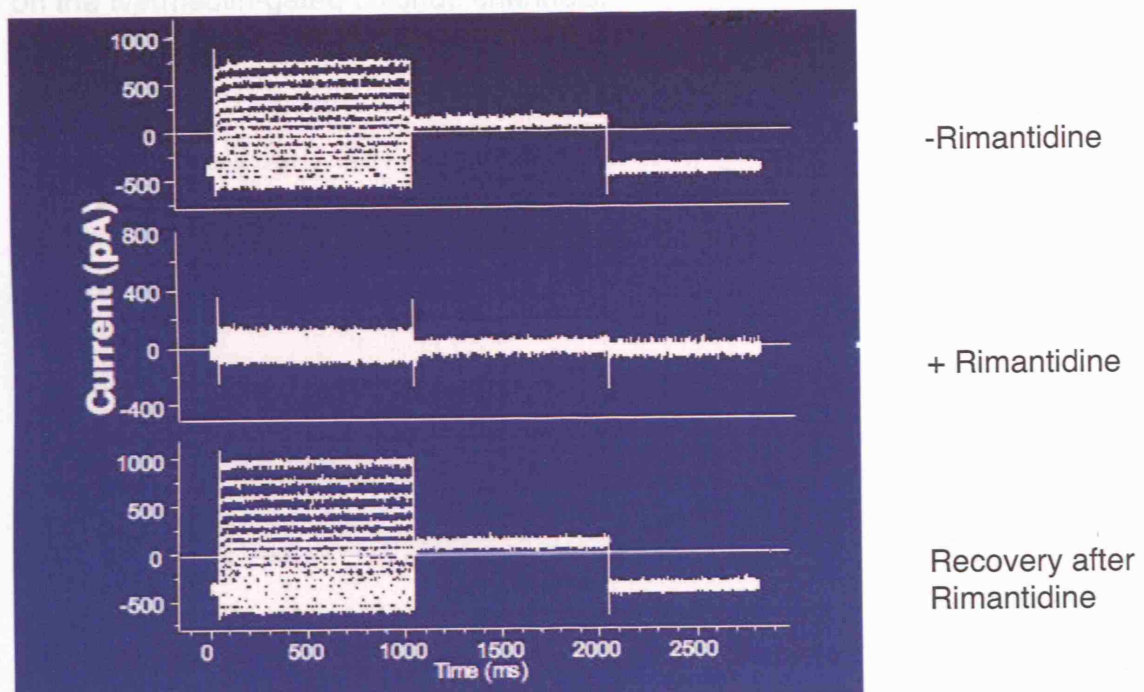
The transgenic mice generated will be used to study interactions of the somatotroph and lactotroph populations following somatotroph ablation and the subsequent effects of Rimantadine treatment. This will be done by detailed imaging analysis, using confocal and 2-photon microscopy, of the remaining somatotroph network in GH-M2 mice crossed to the GH-GFP strain. GH-M2 mice can also be crossed to Prl-DsRed mice to carry out similar analysis of the lactotroph population. A triple cross of GH-M2, GH-GFP and Prl-DsRed will enable interactions of the two networks to be imaged. All transgenic imaging will be compared to mice containing the fluorescently-tagged hormone but which are negative for M2. It will also be possible to study the physiological effects of induction of GH deficiency, upon removal of Rimantadine, at various stages of development.

to analyse the effects of GH-M2 expression on the mouse and its activity by

Figure 1.3 Voltage-clamp assay of GC cells transfected with GH-M2

(from Le Tissier et al., 2005, with permission). GC cells transfected with GH-M2 were assayed by voltage clamp in the absence and presence of Rimantadine. Recordings were inhibited by Rimantadine but returned following removal of the drug.

investigate alternative methods for somatostatin ablation or silencing to study somatostatin and luteinising hormone (Chapter 6). This work is based on the luteinising hormone-gated chloride channels.



The aims of this thesis can be summarised as follows:

- to generate a transgenic mouse line expressing ^{H37A}M2 from the hGH LCR (GH-M2) and to characterise the effects of M2 expression on growth and GH (Chapter 3)

- to analyse the effects of GH-M2 expression on the mouse anterior pituitary by hormone RIA (Chapter 4)
- to image the extent of somatotroph ablation in the anterior pituitary using 2-photon microscopy and analyse the effects of this on the cell network (Chapter 5)
- to investigate alternative methods for somatotroph ablation or silencing to study somatotroph and lactotroph interactions (Chapter 6). This work is based on the Ivermectin-gated chloride channels.

2. MATERIALS AND METHODS

2.1 PRODUCTION OF TRANSGENICS

2.1.1 GH-M2 Cloning

The GH-M2 cosmid was generated by Paul Le Tissier. Cosmid DNA was cut with *NotI* (Roche) and the insert purified by ultracentrifugation through a salt gradient before resuspending in injection buffer (10mM Tris pH 7.4, 0.1mM EDTA) at approximately 20ng/ μ l.

2.1.2 GluCl- α and - β Cloning

Clones encoding GluCl α and GluCl β fused with YFP and CFP, respectively, were obtained from HA Lester (Li et al., 2002) in the expression vector pcDNA3.1. The coding sequence was sub-cloned into a cosmid clone containing the GH LCR (hGH/M2) using standard molecular biology techniques. Primers used in the subcloning were obtained from Invitrogen Ltd and are listed below in Table 2.1. The final cosmid constructs were transfected using Gigapack III Gold Packaging Extract (Stratagene). After sequencing, cosmid DNA was cut with *NotI* and the insert purified by ultracentrifugation through a salt gradient and resuspended in injection buffer (10mM Tris (BDH) pH 7.4, 0.1mM EDTA (BDH)) at 20ng/ μ l.

Table 2.1. Sequences of oligonucleotides used for GluCl α and β subcloning (see figure 6.3).

Primer	Direction	Sequence
T7	5' – 3'	TAATACGACTCACTATAGGG
ghnhebgffor		AGATCTTCTGCTAGCCCGGCTGGCATCCCT
gluaf	5' – 3'	GGGTCCTGTGGACAGCTCACCTAGCTGCAA TGGCCACGTGGATCGTG
gluar	3' – 5'	TGCTTGTAAGCCTGCTTCTC
glubf	5' – 3'	GGGTCCTGTGGACGCTCACCTAGGTGCAAT GGCCACCCCCTCCACCTTC
glubr	3' – 5'	AAGTTGCAGCTCTGGTAGTC

2.1.3 Generating transgenic lines

GH-M2, Glu- α -YFP and Glu- β -CFP cosmids, in injection buffer, were transferred by pronuclear microinjection. The embryos were then transferred to female (CBA/Ca x C57BL/10)F1 mice. This work was done by Sarah Johnson and Sophie Wood of the Procedural Services Division, NIMR.

2.1.4 Animal stocks

Mice were bred and housed (type E cages) at NIMR in the Laidlaw Blue animal house. Stocks were maintained according to NIMR regulations with 12hr light/12hr dark cycle. Food and water were given *ad lib* unless otherwise stated.

2.1.5 Genetic crosses

GH-M2 and GHRH-M2 mice were crossed with established GH-GFP and Pri-DsRed mouse lines.

2.1.6 Genotyping – PCR analyses

Ear punches, taken from 2-week-old mice as a means of identification of individuals in a litter, were used to isolate DNA for genotyping. Each sample was digested in 250 μ l lysis buffer (50mM Tris pH 8.0, 100mM NaCl (Sigma-Aldrich), 100mM EDTA, 1% SDS (Bio-Rad), in dH₂O) containing 0.4mg/ml Proteinase K (Roche) at 55°C overnight.

DNA was extracted by addition of 80 μ l saturated NaCl solution to precipitate proteins and cell debris by centrifugation, followed by isopropanol (Fisher Scientific) precipitation of DNA and 70% ethanol (Fisher Scientific) wash to remove any excess salt. DNA was resuspended in 50 μ l molecular biology grade H₂O (Sigma).

DNA (1-2 μ l) was amplified by PCR using 40 cycles of denaturation, annealing and extension conditions of 94°C/40 seconds, 58°C/40 seconds, 72°C/120 seconds, respectively. A 25 μ l reaction volume was used, consisting of 200 μ M dNTP mix (GE Healthcare), 0.2 μ M primers, 1x Reaction Buffer IV (ABgene), 1.5mM MgCl₂ (ABgene), 2U Taq Polymerase (ABgene). Table 4 lists the primers used for the genotyping of the different strains. Primers GH12 and GH13 were used to genotype potential transgenics for GH-M2, GHRH-M2 and

GH-GFP; primers GH12, GH13 and GFP152 were used to genotype potential transgenics for GluCl α and β ; potential Prl-DsRed transgenics were genotyped using mPRLR1, PRL13 and RPRLGF3.

Table 2.2 Oligonucleotide primers used to genotype transgenic mouse lines

Primer	Direction	Sequence
GH12	hGH 5' UTR	AACCACTCAGGGTCCTGTGGACAG
GH13	hGH 3' UTR	ATGATGCAACTTAATTTTATTAGGACAA
GFP152	GFP reverse	CCACTACCAGCAGAACACC
mPRLR1	mPRL 3' UTR	TTCTGGTCTTGAGTAGTCAC
PRL13	rPRL 3' UTR	ACGCGTTGAAAGATGGCCTTTC
RPRLGF3	rPRL 5' UTR	TGACGGAAATAGATGATTGG
DS2S2975f	SCN4A 3' UTR	TGTACCTGTGGCTGTGTGTC
DS2S2975r	SCN4A 3' UTR	AGATACCCAGACACGTGCAT

Products were separated by electrophoresis through a 1.2% agarose gel in 1xTBE (10x stock: 89mM Tris Base, 89mM boric acid, 2mM EDTA) with 5ng/ml ethidium bromide (Bio-Rad) for analysis. Table 3 shows the expected product size for the transgenic mouse lines.

Table 2.3 PCR product size for transgenic genotyping reactions

Transgenic line	Non-transgenic (NT) product size	Transgenic (T) product size
GH-M2 and GHRH-M2	1523bp	724bp
GH-GFP	1523bp	1226bp
GluClα	1523bp	445bp
GluClβ	1523bp	485bp
Prl-DsRed	1600bp	1005bp

2.2 *In vitro* ANALYSIS OF TRANSGENE EXPRESSION

2.2.1 Tissue culture

GC, GH₃ and GH₄C1 cells were cultured in growth medium (GC - DMEM + glutamine, 15% Horse Serum (GIBCO), 12.5% Fetal Calf Serum (HyClone), 1:100 Gentamicin (GIBCO); GH₃ and GH₄C1 - F10 (Ham) Nutrient Mixture + GlutaMax (GIBCO), 15% Horse Serum, 12.5% Fetal Calf Serum, 1:100 Gentamicin). NIH-3T3 cells were maintained in DMEM containing 10% Fetal Calf Serum.

All cell lines were grown in a humidified incubator at 37°C/5% CO₂. Stable cell lines were selected and maintained using Geneticin G-418 (GIBCO). Cell lines stably transfected with GH-M2 were grown in the presence of 23 μ M Rimantadine (Sigma-Aldrich).

2.2.2 Cell transfections

(i) By Nucleofection

Cells were transfected with 2 μ g of DNA using the Amaxa Nucleofector, programme A23. For transfection cells were placed in Buffer V (Amaxa) prior to electroporation. Transfected cells were plated on polylysine (Sigma) -coated coverslips and maintained in growth medium at 37°C/5% CO₂ for the period of the experiment, usually up to 96 hours.

(ii) By Lipofection

Cells were plated 24 hours prior to transfection in 24-well plates on polylysine-coated coverslips. Cells were transfected using Lipofectamine 2000 (Invitrogen) as described in reagent protocol. Briefly, 1 μ g of DNA was diluted in 50 μ l of serum free medium and mixed gently. 2 μ l of Lipofectamine 2000 was diluted in 50 μ l of serum free medium, mixed and incubated at room temperature for 5 minutes. After 5 minutes, the diluted DNA and Lipofectamine 2000 were mixed together and incubated at room temperature for 20 minutes. The DNA-Lipofectamine 2000 complex mix was then added to each well containing cells and medium (500 μ l serum free). To mix, the plates were rocked gently. Cells were incubated at 37°C for 5 hours. The transfection medium was then replaced with growth medium to prevent cell death. Cells were returned to the 37°C incubator for the remainder of the experiment.

2.2.3 Immunofluorescence

Cells (on coverslips) were removed from growth medium and placed in 4% PFA (Sigma) to fix for 15 minutes. This reaction was quenched and the cells permeabilised using 50mM NH_4Cl and 0.02% w/v saponin for 15 minutes. Cells were then placed in PGAS (0.2% gelatin (Sigma) w/v, 0.02% saponin (Sigma), 0.02% sodium azide (BDH)) for 5 minutes before incubating with the primary antibody (in PGAS) for 45 minutes. Primary antibody was removed by washing with PGAS and then cells incubated with secondary antibody for 30 minutes. Cells were washed in PBS followed by dH_2O and mounted using Mowiol (100% (w/v) glycerol (Sigma), 40% (w/v) Mowiol 4-88 (Calbiochem) in ddH_2O), \pm DAPI (4',6-Diamidino-2-phenylindole, dilactate). All incubations were carried out at room temperature and in the dark.

2.2.4 Western Blot Analysis

GC cells were transfected with constructs and cultured for 24 hours. Cells were then harvested with RIPA buffer (50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM PMSF, 1mM EDTA, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail tablet (1x 25U tablet/10ml; Roche)). Samples were stored at -80°C until use.

Loading buffer (25 μl ; 50mM Tris-HCl, 10mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added to each sample. After denaturation at 95°C for 5 minutes, samples were loaded on to a miniature (Biorad) 8% polyacrylamide gel and separated by electrophoresis at 100V in a buffer solution (25mM Tris Base,

250mM glycine (BDH), 0.1% SDS; in dH₂O). Samples were transferred to Nitrocellulose membrane (Hybond) for 2 hours in 1 x transfer buffer (39mM glycine, 48mM Tris Base, 0.04% SDS, 20% Methanol; in dH₂O). Non-specific binding of the antibody to membranes was blocked by incubation in blocking buffer (5% milk powder in TBS-T (0.1% Tween20 in TBS)) before incubating with the primary antibody [rabbit anti-GFP antibody] for 2 hours at room temperature. After washing in TBS-T, membranes were incubated with anti-rabbit HRP-conjugated antibody (1:10000) in blocking buffer for 1 hour at room temperature. Specific antibody binding to membranes was detected by incubation with Supersignal® Chemiluminescent Substrate (Pierce) for 5 minutes before exposing to film.

2.3 TRANSGENIC ANALYSES

2.3.1 Southern blot

Spleens were dissected from Schedule 1 killed mice and digested overnight in 500μl lysis buffer with Proteinase K 0.4mg/ml. DNA was extracted using phenol/chloroform method. Briefly, 500μl of phenol was added to each tube, vortexed, and centrifuged for 10 minutes at high speed. The aqueous layer was removed and added to tubes containing 500μl chloroform (Fisher Scientific). These were then vortexed and separated by centrifugation as before. The aqueous layer was removed, added to new tubes containing 500μl cold isopropanol and vortexed. A thick viscous DNA precipitate formed and this was removed from the isopropanol using a sterile glass rod. Each precipitate was

then washed with 70% ethanol and allowed to dry briefly before resuspending in 100 μ l TE buffer (10mM Tris-Cl, 1mM EDTA).

The DNA concentration was determined by spectrophotometry at 260nm. DNA was then cut overnight at 37°C using the appropriate restriction enzyme (Roche).

The hGH probe was prepared by PCR amplification of DNA from a GH-GFP mouse using primers GH12 and GH14. This was purified by electrophoresis and extraction using Qiagen gel extraction kit according to the manufacturers instructions and the concentration of the purified DNA determined using a NanoDrop Spectrophotometer (NanoDrop Technologies).

DNA fragments were separated by electrophoresis on a 0.8% agarose gel. The DNA was transferred to Hybond-N+ membrane (Amersham) overnight at room temperature in 0.4M NaOH (BDH). After transfer, the membrane was washed in 2x SSC (0.3M NaCl, 0.03M sodium citrate.2H₂O, pH7.0) for 10 minutes to remove excess NaOH before being placed in an oven at 65°C to dry.

Whilst the membrane was drying the probe was labelled for hybridisation using the Amersham Biosciences Ready-to-Go DNA labelling beads protocol. Briefly, 36 μ l dH₂O was added to the bead and placed on ice to rehydrate. 12 μ l DNA probe was denatured at 95°C for 3 minutes and then immediately placed on ice. 4 μ l of ³²P-dCTP (ReadyVue; Amersham) and 10 μ l of the denatured DNA were

added to the Ready-to-Go mix. This was then incubated at 37°C for 15 minutes. 200µl of ssDNA was then added to the labelled probe and any unbound nucleotides removed by centrifugation at 0.8g for 1 minute through a ProbeQuant G50 mini column (Amersham). The probe was then denatured at 95°C for 3 minutes.

The dried membrane was incubated with 40ml prehybridisation buffer (Church buffer - 10% BSA (Sigma), 1mM EDTA, 0.5M NaHPO₄ – pH 7.2, 7% SDS, made up to 1 litre with dH₂O) with 400µl ssDNA to remove any remaining NaOH and to block non-specific binding of the probe. This was added to the blot in a hybridisation tube and rotated in 65°C oven for 15 minutes.

To stop probe denaturing and rehybridising to itself the probe was diluted in the hybridisation tube containing the membrane in 40ml clean Church buffer. The blot and probe were incubated overnight rotating at 65°C.

The membrane was then washed three times for 15 minutes in 1xSSC then two times 15 minutes in 0.1xSSC. Initially the membrane was exposed to film for 4 hours at room temperature, with subsequent further exposure for a longer period of time if required. The membrane was then exposed to a phospho-image screen (Amersham) for quantification with a scanner and the ImageQuant computer package.

2.3.2 rt-PCR

Tissues were dissected from both transgenic and non-transgenic mice following Schedule 1 kill. Approximately 100mg of each tissue was homogenised using sterile blades and a blunt needle in 1ml of Trizol (GIBCO), 0.2ml chloroform added, the tube vortexed for 15 seconds and incubated at room temperature for 3 minutes. The samples were then spun in a centrifuge at 15.7g for 15 minutes. The aqueous phase was then transferred to a clean tube and RNA precipitated by addition of 0.5ml of isopropanol. Tubes were vortexed and incubated at room temperature for 10 minutes before being spun at 15.7g for 10 minutes. The supernatant was removed and the pellet washed in 1ml 70% ethanol followed by centrifugation at 15.7g for 5 minutes. The RNA pellet was then air-dried at room temperature for approximately 5 minutes after removal of the supernatant. The pellet was resuspended in 10 μ l dH₂O by repeat pipetting and incubation at 55°C for 10 minutes to fully dissolve the RNA pellet. RNA was stored at -80°C until use.

mRNA was transcribed using the SuperScript III cDNA synthesis kit (Invitrogen) and the protocol followed as described. Briefly, 1 μ l of 50 μ M oligo(dT)₂₀, 1 μ l RNA, 1 μ l 10mM dNTP mix and 10 μ l dH₂O were added to a clean microcentrifuge tube. This was mixed by gently tapping the tube and then divided into two to allow for the generation of a negative control sample without addition of reverse transcriptase. The tubes were then incubated at 65°C for 5 minutes followed by 1 minute on ice. To each tube 2 μ l of 5x First Strand Buffer, 0.5 μ l 0.1M DTT, 0.5 μ l RNase inhibitor and 0.5 μ l SuperScript III (to positive

tubes only) were added. This was mixed by gently pipetting up and down and incubated for 30 minutes at 50°C followed by 30 minutes at 55°C. The reaction was inactivated by incubating the samples at 70°C for 15 minutes.

cDNA was amplified using the same reaction mix and amplification cycles as for PCR-genotyping analysis, replacing 1 µl of genomic DNA with 1 µl of cDNA (or negative control). PCR products were separated by electrophoresis on a 1.2% agarose gel and image taken.

2.4 IMAGING TRANSGENE EXPRESSION *ex vivo*

2.4.1 Immunocytochemistry of embedded sections

Mice were anaesthetised and perfused with saline followed by 4% PFA in PB (0.1M Na₂HPO₄, 0.1M NaH₂PO₄, in dH₂O). Pituitaries were dissected and stored in 4% PFA for 2 hours at 4°C before transferring to 70% ethanol. Pituitaries were embedded in paraffin and sectioned by Wendy Hatton, Histology, NIMR. 6 µm sections were cut and stored at 4°C until use.

Sections were placed in Histoclear (National Diagnostics) for 5 minutes to remove paraffin, followed by rehydration in descending concentrations of ethanol (2 minutes each in 100%, 70%, 30%, dH₂O) and finally TBS (50mM TRIS, 0.85% (w/v) NaCl). To ensure antigen retrieval, sections were incubated in 0.05% pepsin (Sigma) in 10mM HCl at 37°C for 7 minutes, washed 2x 5 minutes in TBS to quench the reaction and then incubated in 0.5% TritonX-100 (Sigma) at room temperature for 15mins. After washing in TBS, non-specific

binding was blocked by incubating sections for 1 hour at room temperature in blocking solution (5% BSA, 10% donkey serum (Sigma) in TBS). The primary antibody was diluted in blocking solution and incubated with sections, shaking, at 4°C overnight.

After overnight incubation, unbound primary antibody was removed by washing with TBS 3x 5 minutes. Sections were incubated with secondary antibody at 37°C for 45 minutes. Sections were then washed in TBS to terminate antibody reaction. To mount, sections were washed in dH₂O, dried and then mounted under coverslips with Mowiol + DAPI. Images were taken using epifluorescent and confocal microscopy.

2.4.2 Immunocytochemistry of floating sections

Mice were anaesthetised and perfused as described before. Pituitaries were dissected and kept at 4°C in 4% PFA for 2 hours before transfer to 1xPBS. Pituitaries were embedded in a 4% Agar (Sigma) block, and the block placed in PBS until use. Prior to sectioning the pituitary-containing block was attached to a larger segment of agar to aid positioning. This was then mounted on the cutting block with superglue.

A Leica VT 1000S vibratome was used to cut sections. The chamber was filled with cold PBS. Sections were cut at 40µm and collected in 1x PBS before storing at 4°C until use.

Sections were washed for 5 minutes in PBS before incubating with the primary antibody for 48 hours at 4°C, shaking. The primary antibody was diluted in blocking solution (2% BSA, 2% donkey serum, 0.1% Triton in PBS). Unbound primary antibody was washed off using PBS for 3 x 15 minutes. Sections were then incubated with secondary antibody, diluted in blocking solution, at 4°C in the dark for 2 hours, shaking.

Sections were then washed 3 x 15 minutes in PBS to remove the excess antibody. To stain the nuclei, sections were incubated for 3 minutes at room temperature in Hoechst, diluted 1:250 in PBS. A further 3 washes were used, as before, to remove excess stain. To mount, sections were placed on the slide, dried and then mounted under coverslips with Mowiol. Images were taken using a confocal microscope.

2.4.3 In situ hybridisation

Pituitaries were dissected following Schedule 1 kill and embedded by freezing in O.C.T. (Optimal Cutting Temperature) Compound (Tissue-Tek). 8 µm sections were cut using a cryostat (Leica). Sections were mounted on gelatin-coated slides, prepared as follows. Slides were washed in distilled water containing detergent (Teepol, BDH). To remove excess detergent slides were washed by passing through 8 sequential troughs of distilled water. The slides were then dipped in a gelatin solution (2.5M gelatin, 0.25M chromic potassium sulphate, in DEPC-treated water) and left to dry overnight. Slides were dipped

again on the second day and left to dry overnight. Once dry the slides were stored at -20°C until use.

mGH and M2 DNA was prepared by midi-prep (Qiagen) from bacterial culture. 1 µg of DNA was then digested for 90 minutes at 37°C with restriction enzymes to create sense (T7) and antisense (SP6) forms of the DNA. The DNA was purified as follows. 280 µl of TE, 0.75 µl glycogen and 300 µl phenol/chloroform/isoamylalcohol were added to the cut DNA and vortexed for 10 seconds. The aqueous and organic phases were then separated by centrifugation at 16.1g for 5 minutes. The aqueous phase was then removed and added to a fresh tube containing 300 µl chloroform/isoamylalcohol. The tubes were vortexed and centrifuged at 16.1g for 1 minute. The aqueous phase was again removed and added to a fresh tube to which 15 µl 4M NaCl and 750 µl 100% ethanol was added. Tubes were inverted several times to mix the contents and then allowed to precipitate at -20°C for 1 hour. The precipitate was then separated by centrifugation at 16.1g for 20 minutes at 4°C. The supernatant was removed and the pellet resuspended in 4.5 µl of dH₂O. 0.5 µl of the purified DNA was then run on a 1% agarose gel to check the probe is linear. The remaining purified DNA was stored at 4°C until ready to use.

Two methods of riboprobe detection were used:

(1) Radiolabelled transcription

The cut DNA (2 µl) was mixed with 2.2 µl each of ATP, CTP, GTP and UTP (100mM; Roche), 0.44 µl of 1M DTT, 4.4 µl of transcription buffer (Roche), 8 µl of

^{35}S -UTP (37MBq; Amersham), and $2\mu\text{l}$ of RNA polymerase (1000U; Roche). This mixture was incubated at 37°C for 1 hour. This was then mixed with $4.5\mu\text{l}$ tRNA, $1.5\mu\text{l}$ RNase-free DNase I (1000U; Roche) and $1.5\mu\text{l}$ RNase inhibitor (2500U; Promega) and incubated at 37°C for 15 minutes. Tubes were then heated to 65°C for 5 minutes and cooled on ice for 2 minutes. The volume in each tube was made up to $100\mu\text{l}$ with TE. Unincorporated radiolabel was removed using Pharmacia Nick G50 columns. $1\mu\text{l}$ of each fraction collected was counted for ^{35}S activity.

Sections were fixed in 4% PFA for 5 minutes. After washing in PBS, slides were incubated in triethanolamine (1.4% v/v)/acetic anhydride (0.25% v/v) solution (in sterile saline) for 10 minutes. The sections were then dehydrated by passing slides through a series of alcohol (70-100%) and chloroform dips.

$1 \times 10^6\text{cpm}$ of probe was used per slide. To this $2\mu\text{l}$ of nucleic acid mix ($250\mu\text{l}$ single stranded salmon sperm DNA ($25\mu\text{g}/\text{ml}$), $250\mu\text{l}$ of tRNA ($250\mu\text{g}/\text{ml}$), $500\mu\text{l}$ dH_2O) was added before heating to 65°C for 5 minutes then cooling on ice for 2 minutes. $1\mu\text{l}$ of 5M DTT, $0.5\mu\text{l}$ of 10% SDS and $42\mu\text{l}$ of hybridisation buffer (1M Tris pH7.4, 0.25M EDTA pH8.0, 4M NaCl, 24% (v/v) 50% dextran sulphate, 2.4% (v/v) 50x Denhardt's, 59.5% (v/v) deionised formamide (Sigma), 2.5% dH_2O) per slide was then added. $45\mu\text{l}$ of the mix was applied to each slide, covered with Parafilm, and incubated at 45°C overnight.

Slides were washed in 3 changes of 2 x SSC, followed by two 15 minute washes in 2 x SSC/50% formamide at 45°C. The slides were then dipped in 2 x SSC at 37°C before placing in ribonuclease solution (10x buffer: 1M Tris pH 7.5, 0.5M EDTA pH 8.0, 5M NaCl) containing RNase A (25µl per 50ml; 40mg/ml) for 30minutes at 37°C. Following this incubation, slides were dipped in 2 x SSC at 37°C and washed three times in 2 x SSC/50% formamide at 45°C for 15minutes followed by two washes in 2 x SSC at room temperature for 5minutes. Slides were then rinsed in dH₂O and ethanol and allow to air dry. Slides were exposed to film (BioMax MR; Kodak) for 3-15 days.

(2) DIG-labelled transcription

The protocol followed was as described in product description (Roche). 1µg of linearised DNA, 2µl of DIG RNA labelling mix (10x), 2µl of transcription buffer (10x), dH₂O to 18µl and 2µl of RNA polymerase were mixed and incubated at 37°C for 2hours. The reaction was stopped by addition of 2µl of 0.2M EDTA (pH 8.0).

Slides were fixed for 10 minutes at room temperature in fixative (11.1% (v/v) 36% formaldehyde, 4% (v/v) 10x PBS, in DEPC dH₂O) and then washed 3x 5minutes in 1x PBS. After fixing sections were incubated with triethanolamine/acetic anhydride solution for 10minutes at room temperature. Slides were then washed in 1x PBS as before.

Sections were incubated in hybridisation buffer (50% (v/v) formamide, 5x SSC, 250 μ g/ml tRNA, 5x Denhardt's, 25 μ g/ml salmon sperm, in DEPC dH₂O) for 2 hours at room temperature. During this time, the probe was diluted at 100mg/ml in hybridisation buffer, allowing 100 μ l for each slide. The probe was denatured at 75°C for 10minutes followed by 5minutes on ice. After 2 hours, 100 μ l of the probe mix was added to each slide, covered with coverslips, and incubated at 70°C overnight in a humidified chamber.

Slides were washed in 5x SSC for 5minutes at room temperature, followed by 2 washes of 30minutes at 70°C in 0.2x SSC. They were then washed in 0.2x SSC for 5minutes followed by 5minutes in buffer 1 (0.1M Tris-HCl pH7.5, 0.15M NaCl, in dH₂O), both at room temperature. 800 μ l of blocking solution (10% goat serum in buffer 1) was then placed on each slide for 1hour. Sections were then incubated with 100 μ l of anti-digoxigenin antibody (diluted 1:2500 in blocking solution) overnight at 4°C.

Slides were then washed 3 times in buffer 1 for 5 minutes before being rinsed twice in 2x NTMT (0.2M NaCl, 0.2M Tris pH 9.0, 0.1M MgCl₂, 0.2% Tween) for 10minutes. The immunostaining was then developed at room temperature by adding 800 μ l of staining mix (3.5 μ l nitroblue tetrazolium chloride (NBT; Roche), 3.5 μ l 5-bromo-4-chloro-3-indoyl-phosphate (BCIP 4-toluidine salt; Roche), 5% PVA, in 1x NTMT) to each slide. Slides were incubated in the dark in a humidified chamber. For mGH probe development was approximately 30

minutes and for M2 probes approx. 5 hours. Images were taken using a brightfield microscope.

2.4.4 2-photon imaging

Mice were anaesthetised and perfused with saline followed by 4% PFA in PB. Pituitaries were dissected and stored in 4% PFA for 2 hours at 4°C before transferring to PB containing 0.01% sodium azide. For mice under 21 days of age pituitaries were dissected following killing by a Schedule 1 method and immersed in 4% PFA on ice for 90 minutes. All pituitaries were stored in PB/azide at 4°C. Whole pituitaries were mounted in Mowiol between a 'Parafilm bridge'.

Images were acquired as described by Bonnefont *et al.* (2005). Briefly, a Zeiss LSM 510 NLO confocal system was used. To achieve multiphoton excitation a Chameleon pulsed Ti/sapphire laser (Coherent) was tuned to 840-860nm, at maximum power of approximately 2270-2140mW respectively. The emitted fluorescence was recorded at 500-550nm for GH-GFP and 565-615nm for Prl-DsRed. All images were acquired using x25 oil immersion objective (numerical aperture = 0.8) and an x-y scan speed of 7 (1 pixel/1.60 μ sec; average =2). Images were deconvoluted using Huygens Deconvolution Software (Scientific Volume Imaging) and analysed using Volocity version 4.1 (Improvision).

120 μ m floating sections were cut using the vibratome. Sections were incubated for 10 minutes in Hoescht (1:250 in PBS) in the dark at room temperature. This

was followed by three 15minute washes in PBS before mounting in Mowiol. Pituitary-scale images were taken using the 2-photon set-up described above and the LSM 510 Meta programme stage and scan controls. Images were re-constructed using Image J software (Wright Cell Imaging Facility).

2.5 HORMONE ANALYSES

2.5.1 Tissue collection and preparation

Mice were terminally anaesthetised and decapitated. Blood was collected immediately into tubes containing 5 μ l of heparin (LEO Pharma). Pituitaries were then dissected. Plasma was separated from the total blood sample by centrifugation at 2000g for 10 minutes at 4°C. Plasma was collected and stored at -80°C. Pituitaries were homogenised on ice in 1ml cold PBS. Homogenates were stored at -20°C.

2.5.2 Hormone iodination

Hormone iodination was performed using the iodogen method (Salacinski et al., 1981). Sephadex G75 (Amersham Biosciences) columns were prepared to enable separation and elution of the iodinated hormone from unbound I-125. 0.6 x 25cm columns were used. Prior to use the columns were equilibrated with PBS/BSA (0.3% w/v BSA).

Hormones, kindly provided with standards by A.L. Parlow and the NHPP, were stored at -20°C in 0.2M sodium phosphate solution (pH 7.4) in 10 μ l aliquots. One aliquot was used for each iodination. Immediately prior to iodination a

further 10 μ l of 0.2M sodium phosphate solution was added to the hormone. The Iodine-125 (I^{125} ; 74MBq; Amersham) was stored at +4°C in aliquots of approximately 20MBq (approx. 5 μ l). One aliquot was used per iodination. This was diluted by addition of 15 μ l of 0.2M sodium phosphate solution immediately prior to use.

The hormone was added to the diluted I^{125} . This mixture was then placed in a glass tube containing 5 μ g of dried iodogen (1,3,4,6-Tetrachloro-3 α ,6 α -diphenylglycouril; Sigma), mixed and incubated at room temperature for 5 minutes. 200 μ l of 0.2M sodium phosphate solution was then added and incubated for a further 2 minutes. This mixture was then added to the top of the column and allowed to enter the matrix. The I^{125} -labelled hormone was eluted from the column using PBS containing 0.3% w/v BSA. 1ml fractions were collected manually and MBq counted. The middle fraction with the highest MBq count contains the labelled hormone. This fraction was divided in to aliquots and stored at -20°C until use.

2.5.3 Standard curve

The standard curve was assayed in triplicate. PBS/BSA (0.3% w/v BSA) was used as buffer for the assay. Total counts (T), antibody blank (ABL) and non-specific binding (BO) were used as controls for the assay:

- T - 100 μ l iodinated hormone (diluted to give 5000-10 000cpm) only
- ABL - 200 μ l PBS/BSA + 100 μ l iodinated hormone
- BO - 100 μ l PBS/BSA + 100 μ l antibody + 100 μ l iodinated hormone

Standards were set up as 1:2 serial dilutions from 10ng hormone in PBS/BSA with a final volume of 100 μ l per tube. 100 μ l of antibody solution and 100 μ l of iodinated hormone were then added to each tube. Tubes were then incubated at room temperature for 24 hours. After incubation the bound hormone fraction was precipitated using 18% PEG solution (1 part Tris buffer pH 8.4, 2 parts 27% PEG (Fisher Scientific), 0.02% Triton, 1.5mg/ml bovine gammaglobulin (Sigma)). 600 μ l of 18% PEG solution was added to each tube, except T, and vortexed. Tubes were allowed to stand at room temperature for 30 minutes. Separation was completed by centrifugation at 2000g for 10 minutes at 4°C. The supernatant was then aspirated and the tubes capped. A gammacounter was then used to count the pellets in each tube. Counts were averaged over 3 minutes. % Bo was then calculated for the average of each triplicate and a standard curve plotted using RIA plot.

2.5.4 Pituitary samples – GH, PRL, TSH, LH

Samples were run in parallel with the standard curve. 1:2 serial dilutions of the sample were set up in duplicate in PBS/BSA with 100 μ l final volume. Antibody solution and iodinated hormone were added as for standards.

Tubes were then incubated at room temperature for 24 hours. After incubation the bound hormone fraction was precipitated using 18% PEG solution as for standards. % Bo was then calculated for the average of each set of duplicates using RIA plot. % Bo was then correlated to the standard curve to determine

the mean ng of hormone per duplicate. This could then be converted to μg per pituitary. A typical standard curve gave a 50% B_0 value of 0.17 (GH), 0.37 (PRL), 0.28 (TSH), 0.14 (LH). The interassay variability was 11.2%, 12.7%, 11.6%, and 11.8%, respectively.

2.5.5 IGF-1 RIA

In order to separate IGF-1 from its binding proteins, samples were plasma-extracted on the day of assay using a previously established protocol (Ebensperger et al., 1998). Briefly, the samples were diluted 1:4 in acid-alcohol (87.5% ethanol, 12.5% 2M HCl), vortexed, and incubated at room temperature for 30 minutes. These were then separated by centrifugation at 0.8g for 10 minutes at 4°C. The supernatant was removed and mixed 5:2 with 0.855M Tris Base to neutralise the solution. This was vortexed and incubated at -20°C for 1 hour. Following the incubation, samples were separated by centrifugation at 0.8g for 30 minutes at 4°C. 20 μl of the supernatant was then used for the assay and made up to the 100 μl assay volume with PBS/BSA. Tubes were incubated and developed as for pituitary extracts. Results were converted to ng/ml. A typical standard curve gave a 50% B_0 value of 0.09. The interassay variability was 15.9%.

2.5.6 T4 RIA

The Spectria T4 (^{125}I) kit (Orion Diagnostica) was used to assay plasma total T4 levels. This assay uses pre-coated tubes and supplies standards (nmol/L) and tracer with diluent. Normal uncoated tubes were used for total counts. Briefly,

20 μ l of standards or samples were pipetted in to the designated tube. All tubes, standards and samples, were set up in duplicate. Total tubes remain empty at this point. 500 μ l of the diluted ¹²⁵I-T4 was then added to each tube, including totals, and the tubes vortexed. The tubes were then covered with plastic film and incubated for 2 hours at room temperature. After 2 hours the contents of each tube, except the totals, were decanted and the tubes tapped firmly against absorbent paper. The tubes were left upside down on the absorbent paper for 5 minutes and then tapped firmly again. The counts per minute were measured for 3 minutes for each tube using a gamma counter.

The mean count for the zero standard tube (Bo) was calculated. The %(B/Bo) was then calculated for each standard and sample as follows:

$$\% (B/Bo) = [(standard\ or\ sample\ count) \times 100] / \text{mean zero-standard cpm}$$

These results were then plotted in a semi-log format. T4 concentrations of the unknowns could then be obtained by reading from the plot and averaging the duplicate values. T4 values obtained were in nmol/L.

These were converted to ng/ml as follows:

$$T4\ (ng/ml) = T4\ (nmol/L) \times 0.777$$

2.6 ANIMAL TREATMENTS

2.6.1 hGH injection

Twice daily hGH (Humatrope 12U; Eli Lilly) injections were given to high copy line male mice to attempt to improve breeding ability. 25-37.5 μ g/100 μ l injections were given depending on weight of individual mouse to comply with dosage limitations outlined by the Home Office project licence. Mice were weighed at 4 weeks and the daily regimen of hormone calculated accordingly. Mice were weighed daily throughout treatment with hGH. Injections were stopped when mice reached a body weight of >15g.

2.6.2 Estrogen treatment

8-week-old GH-M2 low and medium copy male mice were weighed and arranged into weight-matched groups for treated and untreated, transgenic and non-transgenic groups (n=6). 0.1mg β -estradiol-3-benzoate pellets (Innovative Research of America) were implanted subcutaneously under general anaesthetic. After stitching the incisions mice were given a dose of analgesic (5mg/kg; Rimadyl small animal; Pfizer) and antibiotics (Fucidin; LEO Pharma) to limit pain and aid recovery.

Mice were checked daily and weighed at regular intervals to monitor any adverse effects of the estrogen treatment. At 2 weeks after implantation mice were killed by a Schedule 1 method and the pituitary and blood collected from each animal. These were then analysed by RIA.

2.6.3 Rimantadine treatment

Two different methods of dosing with Rimantadine were used:

1. 42 day old GH-M2 low copy line male mice and their non-transgenic littermates (n=5-6) were weighed and treated with Rimantadine at 1mg/ml in the drinking water for three weeks. Control mice (T and NT) were left untreated. The treated water was changed every 3-4 days and all of the mice weighed. At the end of the period mice were weighed and the pituitary taken after Schedule 1 kill. GH levels were analysed by RIA.
2. 30 day old GH-M2 low copy line male mice and non-transgenic littermates were arranged into weight-matched groups of n=6. Mini-pumps containing 50mg/ml Rimantadine in dH₂O were inserted subcutaneously under anaesthetic. Analgesic and antibiotics were given immediately post-operatively, as previously described, and the health of the mice monitored on a daily basis. Treatment lasted for 14 days. Mice were weighed at the start and end of the experiment and at 3-4 day intervals during the treatment. Control mice were weighed at the same time but were otherwise left untreated. At the end of this experiment, pituitaries were taken after Schedule 1 kill and the GH content measured by RIA.

2.6.4 GHRH treatment

8 week old low copy line male mice and non-transgenic littermates were arranged into weight-matched groups of n=4-6. Mice were anaesthetised by

intraperitoneal injection of pentobarbitone. A canula was inserted in to the jugular vein to aid injection and blood withdrawal. A basal sample of blood was taken. This was followed by injection of GHRP-6 (Ferring AB) (500ng/50 μ l). A blood sample was taken five minutes after injection. After 2 hours a second basal blood sample was withdrawn, followed by injection of GHRH ([Nle²⁷]GRF(1-29)NH₂; Bachem) (100ng/50 μ l). The blood was sampled 5 minutes after injection.

At each sampling timepoint 50 μ l of blood was extracted. Blood samples were immediately diluted in 100 μ l PBS/BSA, mixed and spun in a centrifuge. 100 μ l of plasma supernatant from basal sampling and 20 μ l of plasma supernatant from the secretagogue challenge were assayed by GH RIA and results expressed as ng/ml of plasma.

2.7 STATISTICAL ANALYSES

Results were presented as mean \pm S.E.M. and statistical analysis was carried out in Instat 2.01. Where two groups were compared statistical analysis was performed using the unpaired Students t-test. Where multiple groups were compared analysis of variance (ANOVA) followed by post-testing of selected pairs using Bonferroni's comparison was used to determine the groups showing significant differences. Significance values were assigned: * P<0.05, ** P<0.01, *** P<0.001.

3. CHARACTERISATION OF THE GH-M2 TRANSGENIC MOUSE LINES

3.1 INTRODUCTION

The ^{H37A}M2 variant has previously been targeted *in vivo* to T cells (Smith et al., 2002) and GHRH neurons (Le Tissier et al., 2005) in an attempt to produce a mouse model to study cell function utilising this technique of conditional cell ablation, controllable by addition of Rimantadine, an M2-targeted anti-influenza drug. Treatment of these mouse models with Rimantadine had shown the phenotype resulting from ^{H37A}M2 expression could not be reversed *in vivo*, however. It is hoped that targeting the ^{H37A}M2 channel to the somatotrophs, as described in chapter 1, will produce a more conditional transgenic model for studying pituitary plasticity. The non-reversibility of the phenotype in the transgenic models previously generated with the ^{H37A}M2 channel may not have been possible because of an absence of progenitor GHRH neurons to replace those expressing the transgene. If an adult pituitary progenitor cell population exists the phenotype would be reversible in the presence of Rimantadine. Furthermore, even if it is not possible to reverse the phenotype *in vivo* with Rimantadine treatment it may still be possible to rescue somatotroph function in primary cultured anterior pituitary cells.

Before I joined the lab attempts had been made to generate GH-M2 mice. Previous microinjections of the GH-M2 cosmid yielded one founder male. This mouse was small but sterile and could not be used to establish a line, although some animals might have been obtained with *in vitro* fertilisation (IVF). One

possibility is that this was due to insertional disruption by the transgene of a gene, or genes, whose product was essential for reproductive ability. Alternatively, the transgene could have become fragmented, resulting in misexpression of the protein in tissues where GH is not normally expressed, for example, to the gonadotrophs or cells governing steroid hormone production. Since the line could not be established, no analysis was performed. Instead, I recommenced microinjections at the beginning of my project with freshly prepared GH-M2 cosmid to generate more founders. Three founders were generated and bred to establish these lines. The aim of the work outlined in this chapter was to characterise the transgene expression, phenotypes, and potential reversibility of the phenotype by Rimantadine treatment in the resulting GH-M2 lines.

3.2 EXPERIMENTAL RESULTS

Of the 117 pups born following pronuclear microinjection, three tested positive by PCR for the presence of the GH-M2 transgene. Of these founder mice only one, a female, appeared small. The founders were crossed to wild type mice to test whether the transgene would transmit and to establish the three lines.

3.2.1 Genotyping GH-M2 mice

Ear punches were taken from pups prior to weaning (approximately 21 days). DNA was isolated from the earpieces for PCR amplification.

Figure 3.1A is an example of the PCR used to genotype the GH-M2 line. Primers hGH12 and hGH13 are used to amplify a fragment of the insert (T) (figure 3.1B). These primers also cross react with a fragment of the endogenous mouse GH sequence, to produce a larger PCR product (NT). GH12 is the forward primer and is designed to the 5' untranslated region (UTR) of exon 1. GH13 is the reverse primer and complements the 3' UTR. The transgenic band is 724 bp in size and the endogenous GH band is 1523bp.

3.2.2 Southern blot analysis

Initial observations of the first generation of offspring indicated a growth phenotype difference between the three lines. The transgenic offspring from two of the lines showed no discernable difference to non-transgenic littermates. However, the two transgenic pups born to one of the male founders appeared severely growth retarded.

Analysis by Southern blot was used to estimate the relative transgene copy number of the three lines. DNA was isolated from the spleens of transgenic and non-transgenic littermates from the three lines and compared to DNA isolated from the spleen of a GH-GFP mouse, as a positive transgene control. GH-GFP control DNA was digested with the restriction enzymes *Bgl*II and *Nhe*I. The restriction sites for these enzymes are located outside the transgene sequence, as shown in figure 3.2A(i). Digesting the DNA with these enzymes results in a fragment containing GH sequences and the integration site of the transgene. The intensity of the resulting GH-hybridising fragment is equivalent

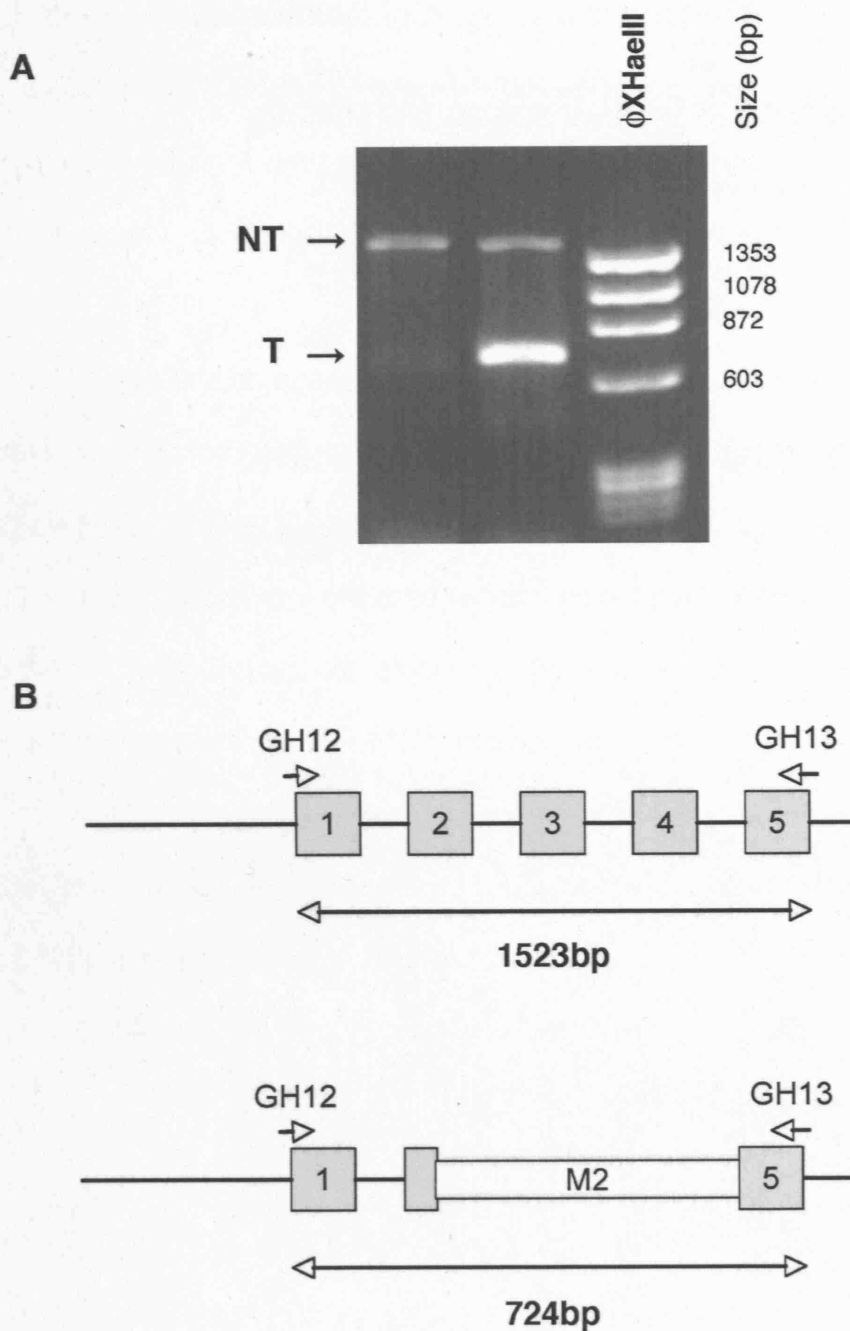


Figure 3.1 Genotyping of the GH-M2 mouse line

(A) PCR analysis of the DNA isolated from a transgenic and non-transgenic mouse ear notch. The transgenic band is 724bp compared to 1523bp for the non-transgenic/endogenous GH band.

(B) Primers hGH12 (forward) and hGH13 (reverse) are used for the genotyping PCR. Numbers 1-5 (grey boxes) refer to the hGH exons.

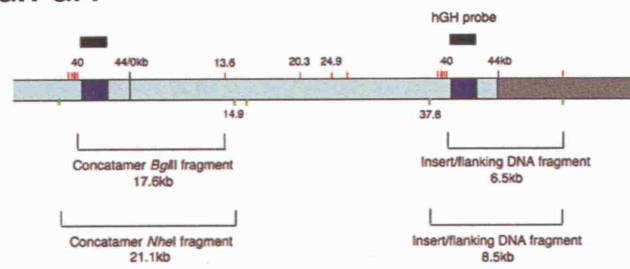
Figure 3.2 Southern blot analysis of the three GH-M2 lines

(A) Restriction map of GH-GFP (i) and GH-M2 (ii). Not to scale. Grey bar = endogenous DNA, red tab = *Bgl*II site, green tab = *Nhe*I site, dark blue box (i) = GH-GFP sequence, red box (ii) = GH-M2 sequence. The arrow indicates the site of SCN4A within the LCR.

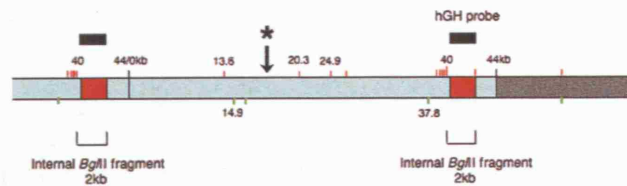
(B) DNA extracted from the spleen of transgenic and non-transgenic mice from the three lines generated by oocyte microinjection, was digested with *Bgl*II and probed with hGH. The intensity of the positive band (2kb) was assessed using a phosphoimager and compared to the intensity of the integration site band of GH-GFP DNA, cut *Nhe*I, to estimate copy number. The intensity of this band correlates to the copy number of the GH-M2 transgene in each mouse line.

(C) PCR amplification of DNA isolated from low, medium and high copy line mice and non-transgenic littermates with SCN4A primer set DS2S2975 (<http://www.ncbi.nlm.nih.gov/genome/sts/sts.cgi?uid=74585>). The arrow indicates the positive band of 152bp.

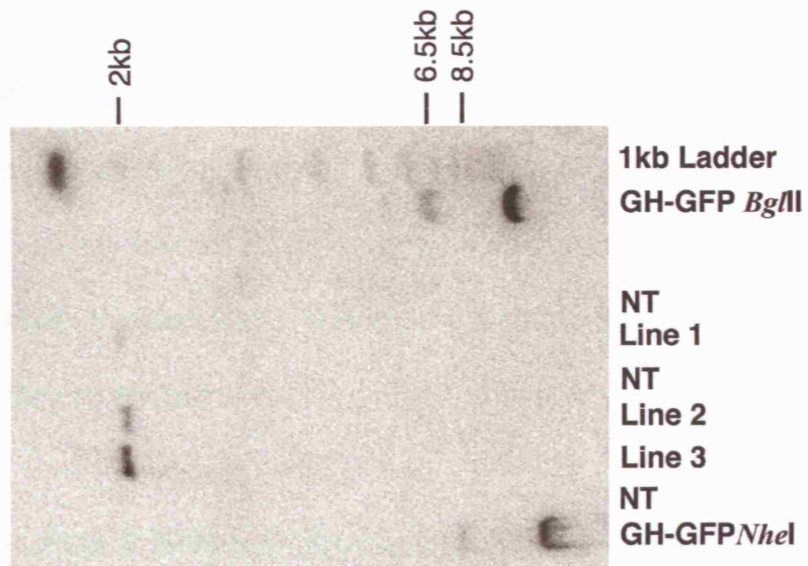
A (i) GH-GFP



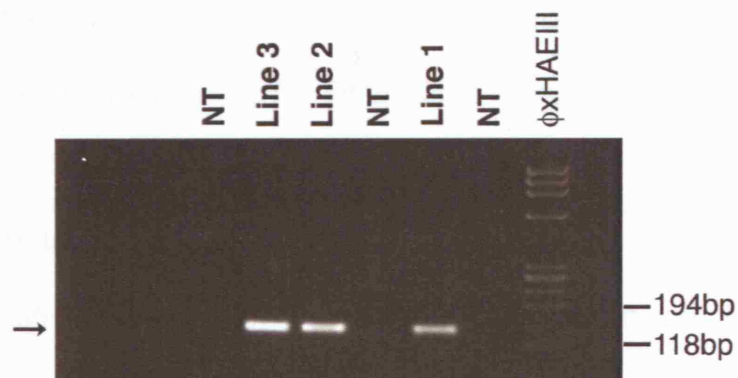
(ii) GH-M2



B



C



to one copy of the transgene. GH-M2 DNA isolated was cut with *Bgl*II. Restriction sites for *Bgl*II flank the GH-M2 sequence (figure 3.2A(ii)) of the transgene to reveal insertion of multiple copies of the transgene. The southern blot was probed with a PCR amplified fragment of hGH labelled with ³²P.

Figure 3.2B shows the Southern blot for DNA from the three GH-M2 founder lines compared to a non-transgenic littermate and a GH-GFP transgenic mouse. The intensity of the band was analysed using ImageQuant software and compared to the smaller sized GH-GFP bands, which is assumed to correlate to 1 copy of the transgene. This analysis indicates that line 1 contains at least 1 copy of the GH-M2 transgene, line 2 contains two times as many copies as line 1, and line 3 contains at least 6 times as many copies. From this point on the lines 1, 2 and 3 will be referred to as the low, medium and high copy lines, respectively, for ease of discussion. This is an estimated quantification due to the limits of accuracy of this technique.

PCR amplification of the 3' UTR of SCN4A was used to confirm the presence of the hGH LCR in the three transgenic mouse lines. SCN4A is situated 5' of the HSI and HSII sites of the hGH LCR. Figure 3.2C shows the PCR amplification in the DNA from low (line 1), medium (line 2) and high copy (line 3) mice indicating that an intact LCR is present in each line (152bp band). This should direct cell specific, copy number dependent, integration site independent expression of the transgene (Jones et al., 1995; Bennani-Baiti et al., 1998).

3.2.3 Growth of transgenic and non-transgenic littermates

High copy line mice are relatively infertile and there were insufficient numbers of animals to gain a complete growth curve. For this line initial weights were taken prior to the use of the mouse for another purpose, such as breeding or pituitary hormone analysis. The average weight of a high copy line mouse at 42 days is approximately 9.1g (NT: $24.6\text{g} \pm 0.48$) and 8.7g (NT: $17.5\text{g} \pm 0.67$) for male and female mice, respectively (data not shown). When 3-week-old high copy line male mice were treated with a twice-daily injection of hGH for 2 to 3 weeks, they showed an increase in body weight (table 3.1). This improved the frequency of litters from some, but not all, breeders.

Table 3.1 hGH-induced growth of high copy line stud males. Mice were treated with hGH from 3 weeks of age and growth monitored. The weight of these high copy line mice before and after treatment is shown, with the subsequent number of litters from each male when paired with a WT female.

Mouse	Weight before (g)	Weight after (g)	Number of litters
1	7.5	16.0	3
2	8.0	15.4	1
3	8.0	15.1	1
4	7.5	14.5	2
5	7.7	15.3	2

Transgenic and non-transgenic littermates of the low and medium copy lines were weighed at weekly intervals from 3 to 8 weeks of age (n=5-8). Figure 3.3

shows the growth curves for male (A) and female (B) GH-M2 low and medium copy line animals compared to non-transgenic littermates. The mean weight at each time point was plotted \pm S.E.M. Male and female low copy line mice exhibit both a similar rate of growth and similar mean body weights to the non-transgenics throughout this time period. Medium copy line male mice have a similar body weight ($12.68\text{g} \pm 0.53$) compared to NT ($12.9\text{g} \pm 0.39$) at 3 weeks prior to the onset of puberty. By 4 weeks of age, however, there is a significant difference in body weight between these two groups of mice; for example, medium copy line male mice have a mean body weight of $15.87\text{g} \pm 0.52$ (NT: $19.23\text{g} \pm 0.98$; $P < 0.01$). At 8 weeks old the medium copy line mice have reached an equivalent body weight (approx 27g), indicating that it is the initial rate of growth that is affected. The decreased body weight observed in medium copy line female mice from 4 weeks compared to non-transgenic littermates is not significant at any age up to 8 weeks.

Figure 3.4A shows mean nose to anus (N-A) lengths for low and medium copy line male mice compared to non-transgenic littermates at 8 weeks. Medium copy line males have a significantly shorter body length of $9.31\text{cm} \pm 0.08$ ($P = < 0.05$) compared to non-transgenic littermates ($9.86\text{cm} \pm 0.12$). This indicates that although medium copy line male mice gain in body mass in a similar way to non-transgenic littermates, longitudinal bone growth is adversely affected. Tail lengths were also recorded at this age (figure 3.4B) but no significant difference was detected between the three groups.

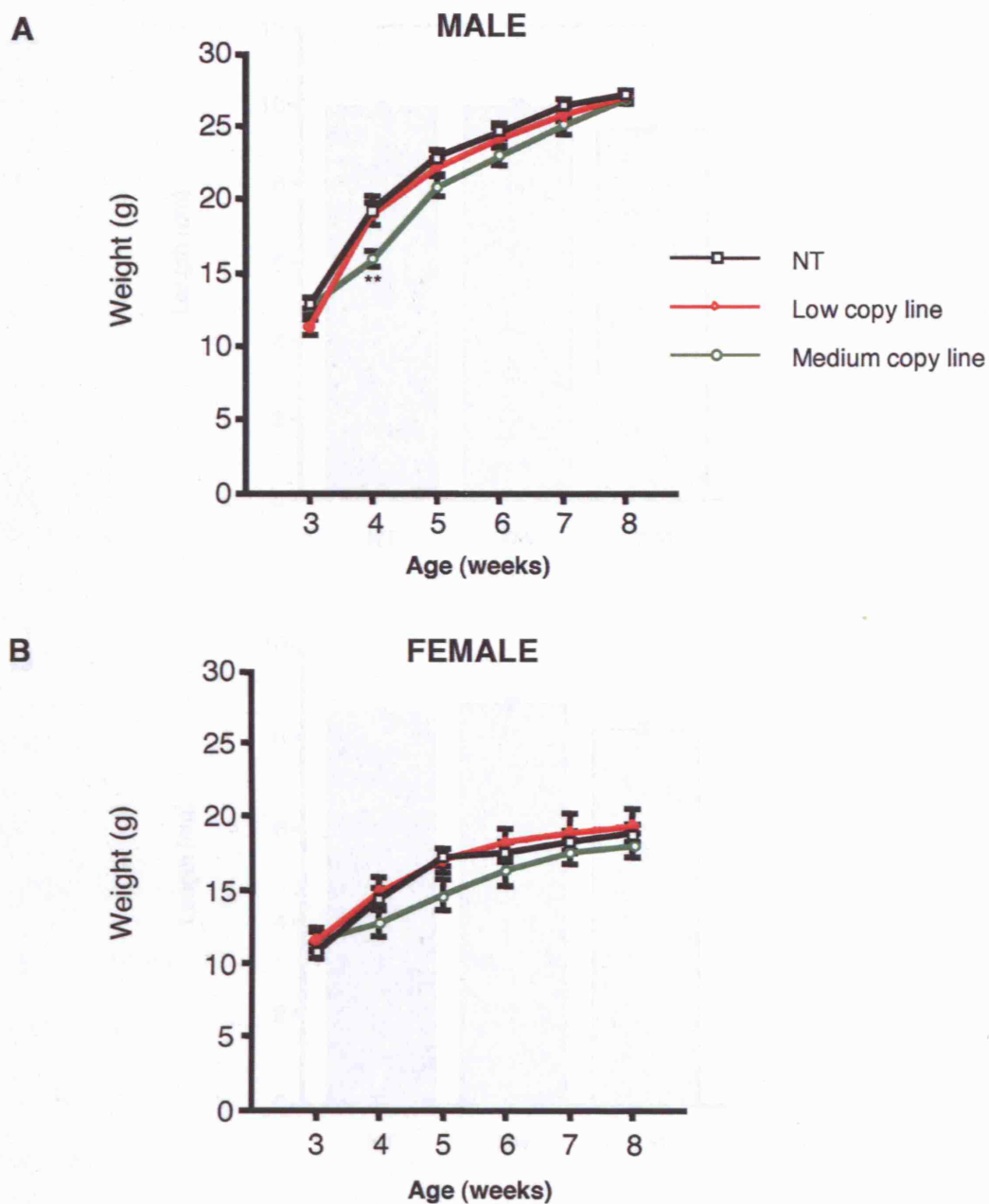


Figure 3.3 Growth curves of male and female low copy, medium copy and non-transgenic mice

(A) Male and (B) female mice from the low copy and medium copy lines were weighed at weekly intervals from 3 to 8 weeks of age and compared NT littermates.

** $P < 0.005$ Transgenic vs non-transgenic. ANOVA followed by Bonferroni's post-hoc test ($n=5-10$).

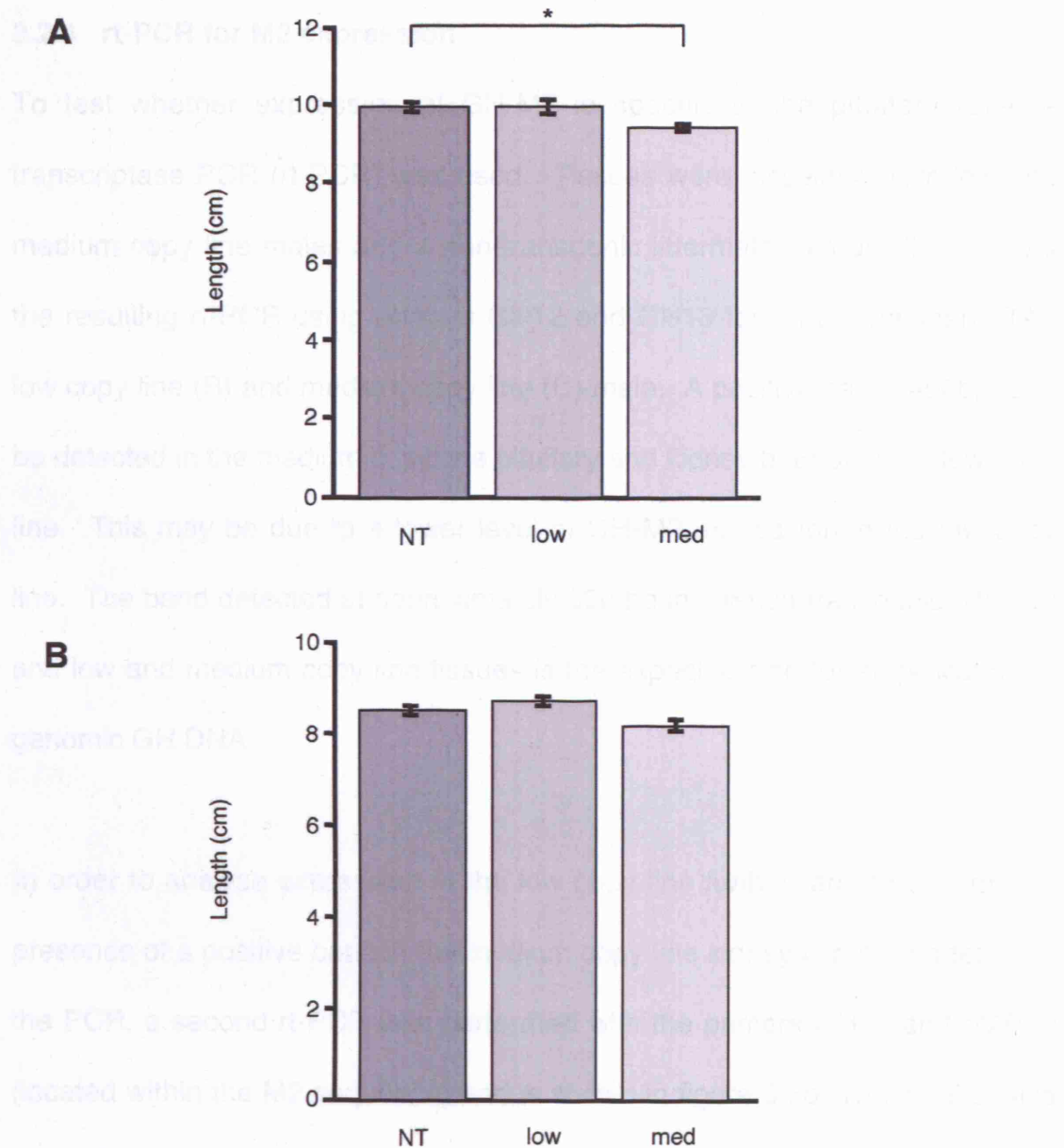


Figure 3.4 Growth measurements of male low copy, medium copy and non-transgenic mice at 8 weeks of age

(A) Nose to anus length

(B) Tail length

* $P < 0.05$ Transgenic vs non-transgenic. ANOVA followed by Bonferroni's post-hoc test ($n=5-10$).

3.2.4 rt-PCR for M2 expression

To test whether expression of GH-M2 is specific to the pituitary reverse transcriptase PCR (rt-PCR) was used. Tissues were dissected from low and medium copy line males and a non-transgenic littermate. Figure 3.5a shows the resulting rt-PCR using primers GH12 and GH13 for a non-transgenic (A), low copy line (B) and medium copy line (C) male. A positive band (450bp) can be detected in the medium copy line pituitary and kidney but not in the low copy line. This may be due to a lower level of GH-M2 expression in the low copy line. The band detected at approximately 800 bp in the non-transgenic pituitary and low and medium copy line tissues is the expected size for amplification of genomic GH DNA.

In order to analyse expression in the low copy line further, and to confirm the presence of a positive band in the medium copy line kidney is not an artefact of the PCR, a second rt-PCR was performed with the primers GH12 and M2Rev (located within the M2 sequence) and is shown in figure 3.5b. The smaller size of the PCR product increases the efficiency of the amplification reaction. With this primer set a positive band (350bp) corresponding to amplification of M2 was detected in the low copy line pituitary and the medium copy line pituitary, kidney and testis. The band at approximately 600bp is thought to correlate to amplification of genomic GH DNA.

Amplification of the ubiquitously expressed hypoxanthine-guanine phosphoribosyl transferase (HPRT) cDNA with HPRT forward and reverse

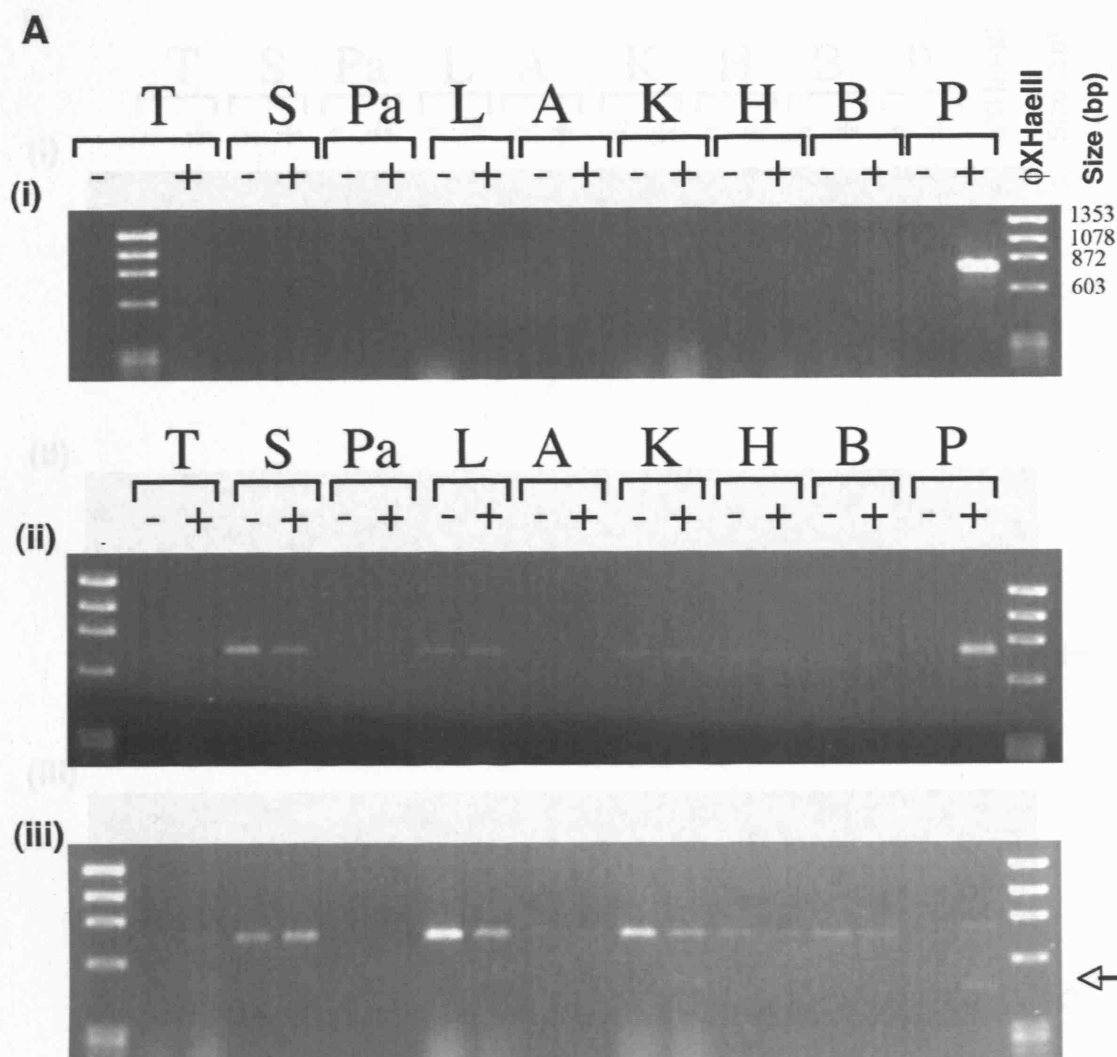


Figure 3.5 RT-PCR of male NT, low and medium copy line tissues

RNA was isolated from a range of mouse tissues from a low and medium copy line mouse and a non-transgenic littermate. cDNA was generated with Reverse Transcriptase and PCR performed on each.

(A) Primer set hGH12/hGH13 was used to amplify the cDNA.

- (i) Non-transgenic
- (ii) Low copy line
- (iii) Medium copy line

T=testis, S=spleen, Pa=pancreas, L=liver, A=adrenal gland, K=kidney, H=heart/lung, B=brain, P=pituitary

Arrow indicates positive band (approx. 450bp)

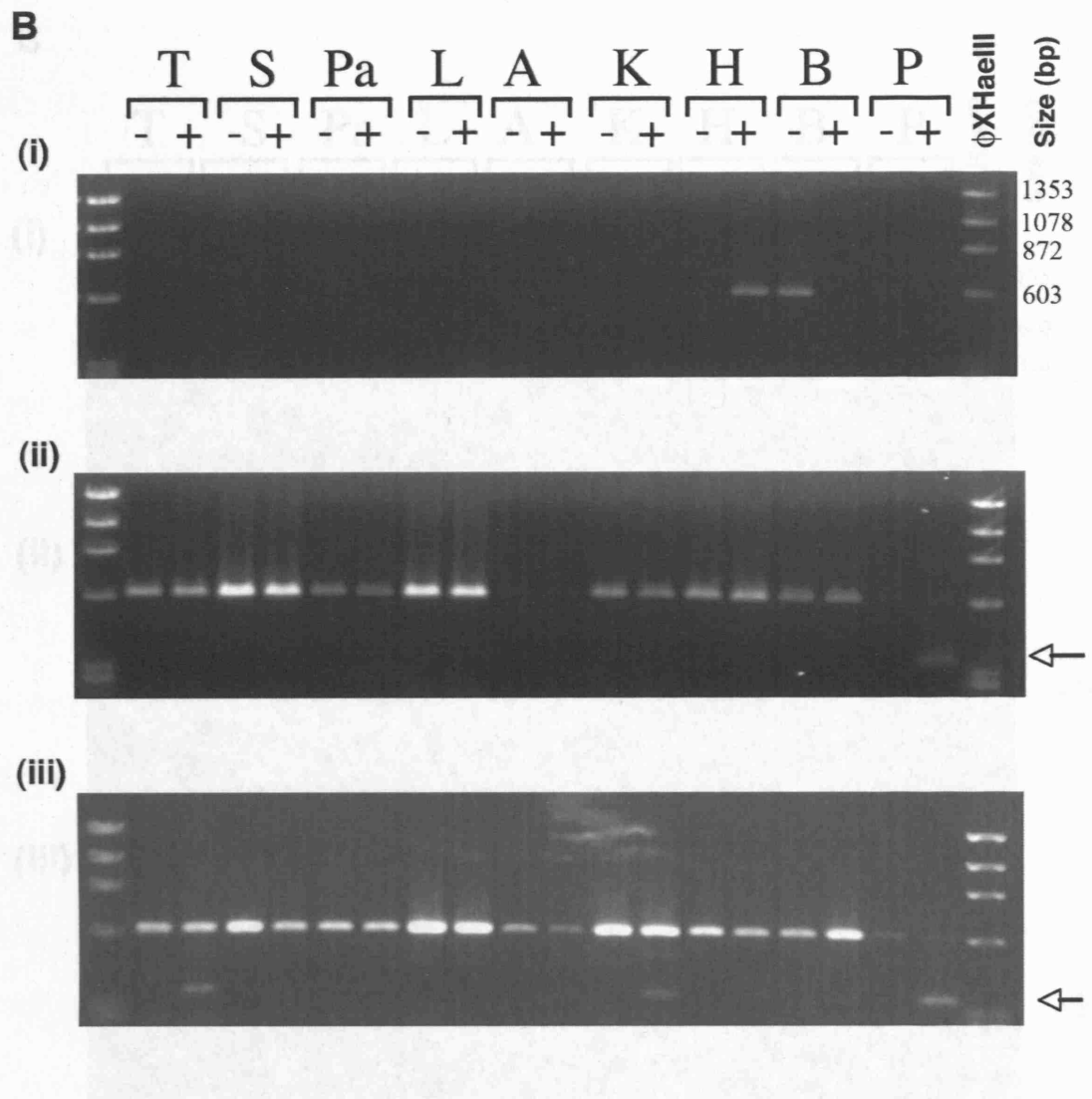


Figure 3.5 RT-PCR of male NT, low and medium copy line tissues

(B) Primer set hGH12/M2rev was used to amplify the cDNA.

- (i) Non-transgenic
- (ii) Low copy line
- (iii) Medium copy line

T=testis, S=spleen, Pa=pancreas, L=liver, A=adrenal gland, K=kidney, H=heart/lung, B=brain, P=pituitary

Arrow indicates positive band (approx. 350bp)

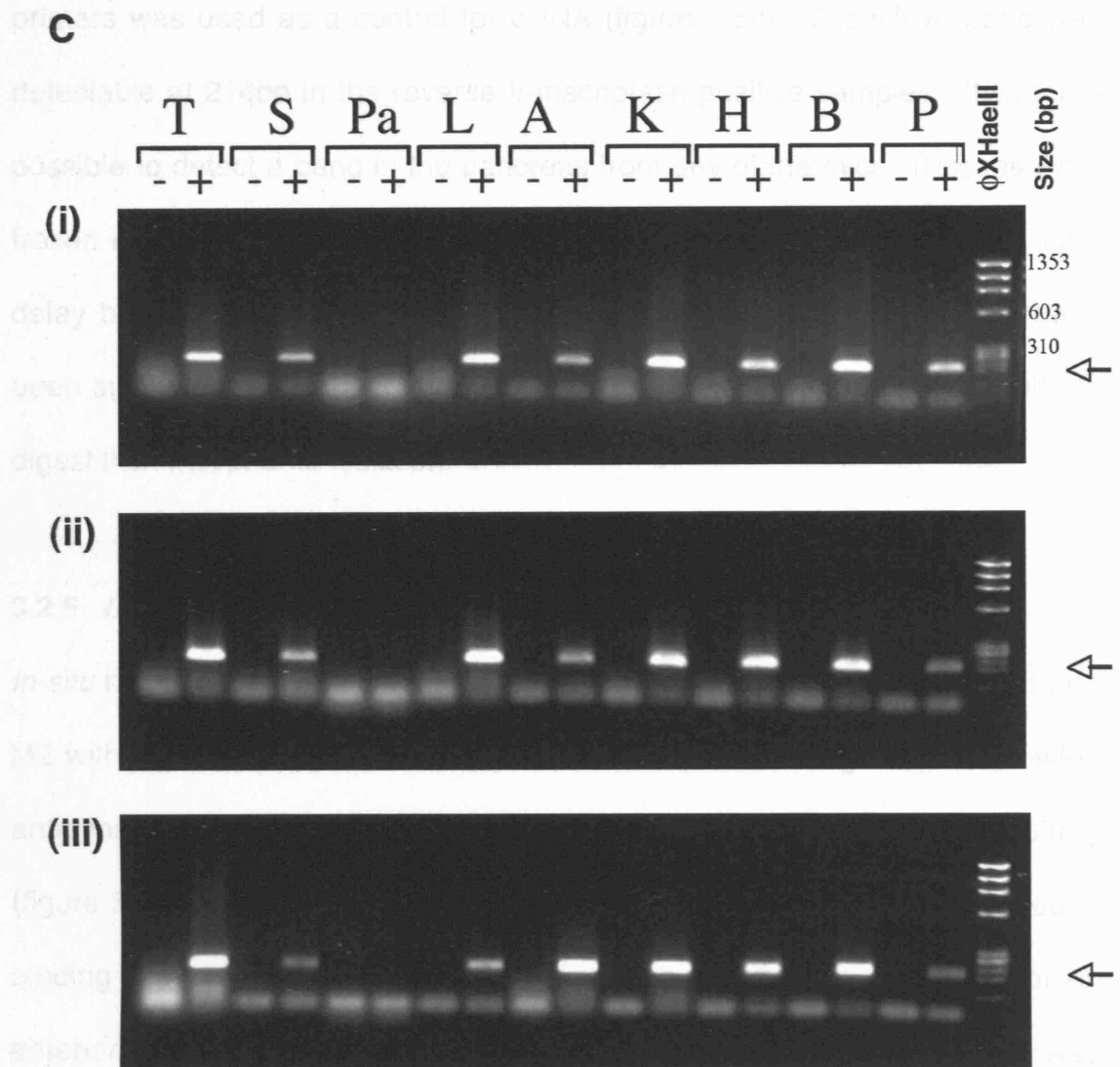


Figure 3.5 RT-PCR of male NT, low and medium copy line tissues

(C) Primer set HPRT forward/reverse was used to amplify the cDNA

(i) Non-transgenic

(ii) Low copy line

(iii) Medium copy line

T=testis, S=spleen, Pa=pancreas, L=liver, A=adrenal gland, K=kidney, H=heart/lung, B=brain, P=pituitary

Arrow indicates positive band (214bp)

primers was used as a control for cDNA (figure 3.5c). A positive band was detectable at 214bp in the reverse transcriptase positive samples. It was not possible to detect a band in the pancreas from any of the mice. Tissues were frozen immediately after dissection and stored at -80°C until use. The time delay between initiating the dissection and freezing the pancreas may have been sufficient for the high level of endogenous pancreatic RNase activity to digest the RNA prior to isolation.

3.2.5 *In situ* hybridisation for GH and M2

In situ hybridisation was used to attempt to measure colocalisation of GH and M2 within the pituitary. GH expression was detected using the DIG-labelled antisense GH probe in both the non-transgenic and low copy line pituitary (figure 3.6A i & iii). Reaction with the sense probe for GH gave no background binding (figure 3.6A ii & iv). As would be expected, a large number, but not all, anterior pituitary cells showed specific hybridisation. A similar result was obtained with the ³⁵S-labelled probe.

Hybridisation with the M2 probe gave a high level of background binding in the non-transgenic pituitary (figure 3.6B i & ii), making it difficult to be sure of specific detection of the M2 transcript. It was not possible to detect positive M2 staining in the transgenic pituitary with the antisense probe (figure 3.6B iii) above the sense background (3.6B iv). It was necessary to incubate the pituitary sections with the M2 probes overnight as no binding could be distinguished after 6 hours. It is possible therefore that this increased the level

of background binding in both the non-transgenic and transgenic pituitaries. Further optimisation of this assay is required. It may also be necessary to identify a second M2 probe, as a different probe may have an increased level of specificity. A similar result was seen with the ^{35}S -labelled M2 probes.

3.2.6 Immunofluorescence for M2 localisation

Due to the difficulties encountered in identifying M2 by *in situ* hybridisation immunofluorescence for GH and M2 (figure 3.7) was used to visualise the localisation of M2 within the pituitary in relationship to somatotrophs in a low copy (D-F) and medium copy (G-I) male at 6 weeks compared to an age-matched non-transgenic male (A-C).

In the non-transgenic control pituitary GH positive cells are detectable by immunofluorescence (figure 3.7A). However, these cells appear irregular in shape suggesting a problem with perfusion fixation of the pituitary. Some non-specific, diffuse binding with the M2 antibody can be detected, as shown in figure 3.7B and C.

GH positive cells can also be detected in the low copy line pituitary (3.7D). These cells are more round in shape than those observed in the non-transgenic pituitary. Immunofluorescence for M2 (3.7E) revealed M2 positive cells in the anterior pituitary of these mice. The majority of this staining appears to be membranous although some more diffuse staining can be observed. When the

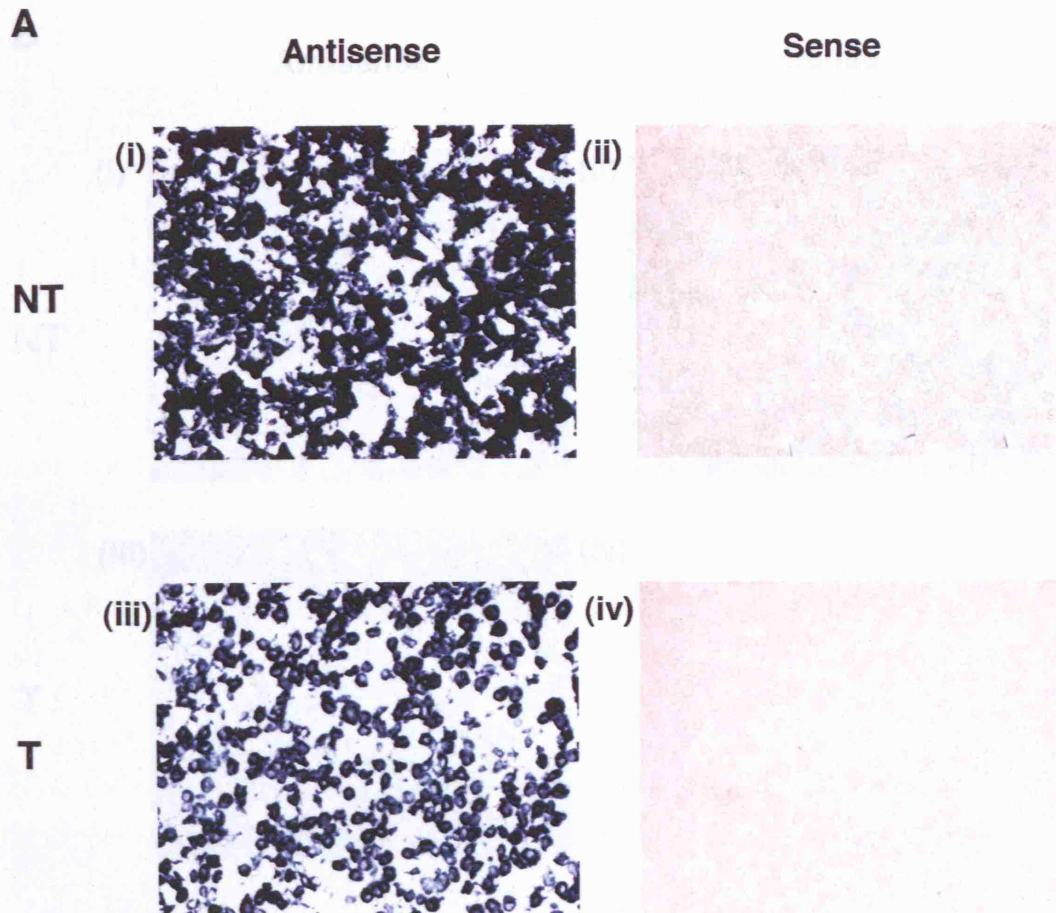


Figure 3.6A *In situ* hybridisation for GH in frozen pituitary sections

DIG-labelled *in situ* hybridisation for GH in a non-transgenic (i, ii) and a low copy line (iii, iv) male pituitary from 8 week old mice. Images were taken using a 40x objective on a brightfield microscope.

- (i) NT pituitary incubated with antisense DIG-labelled probe.
- (ii) NT pituitary incubated with sense DIG-labelled probe.
- (iii) T pituitary incubated with antisense DIG-labelled probe.
- (iv) T pituitary incubated with sense DIG-labelled probe.

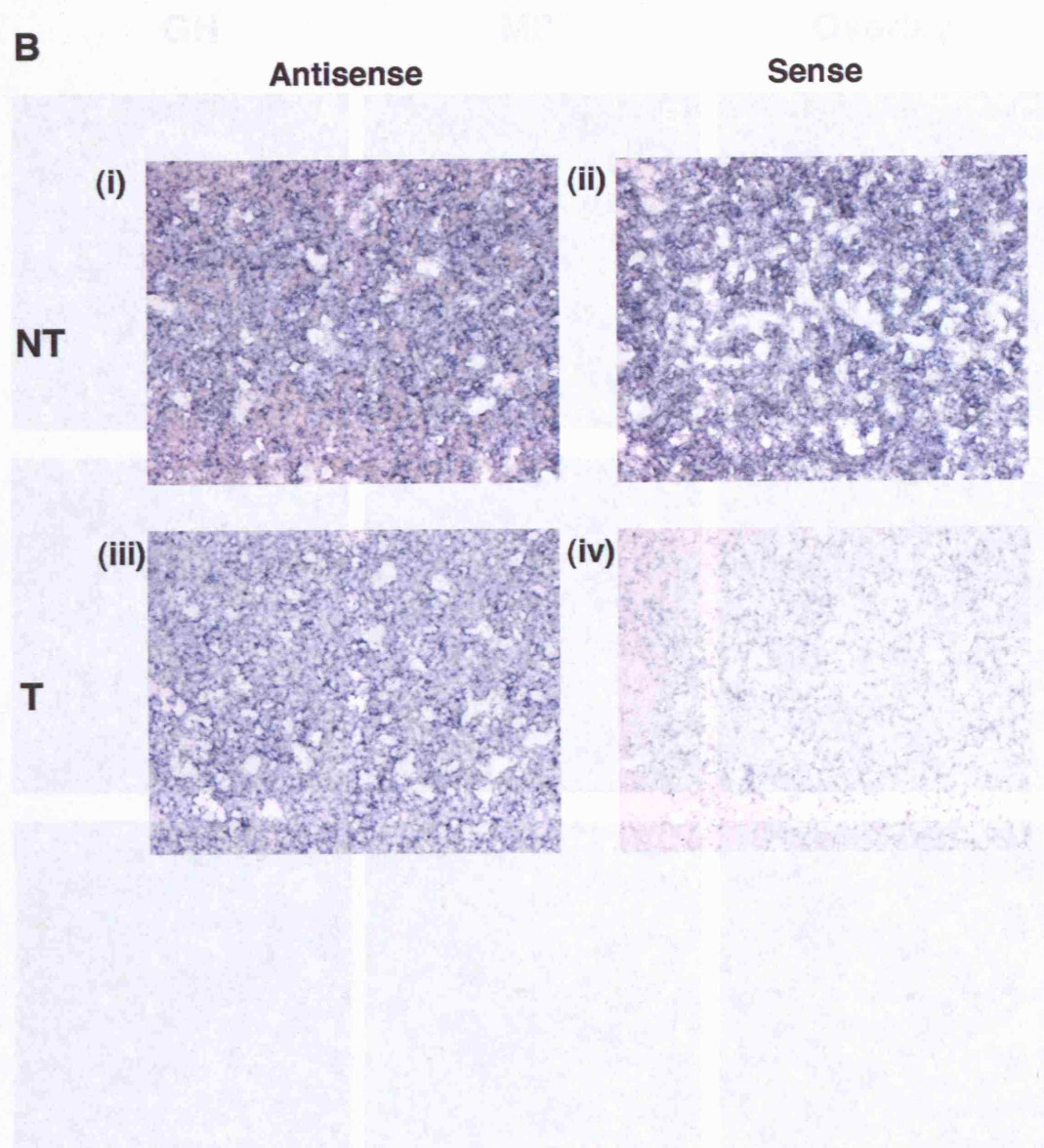


Figure 3.6B *In situ* hybridisation for M2 in frozen pituitary sections

DIG-labelled *in situ* hybridisation for M2 in a non-transgenic (i, ii) and a low copy line (iii, iv) male pituitary at 8 weeks of age. Images were taken using a 40x objective on a brightfield microscope.

- (i) NT pituitary incubated with antisense DIG-labelled probe.
- (ii) NT pituitary incubated with sense DIG-labelled probe.
- (iii) T pituitary incubated with antisense DIG-labelled probe.
- (iv) T pituitary incubated with sense DIG-labelled probe.

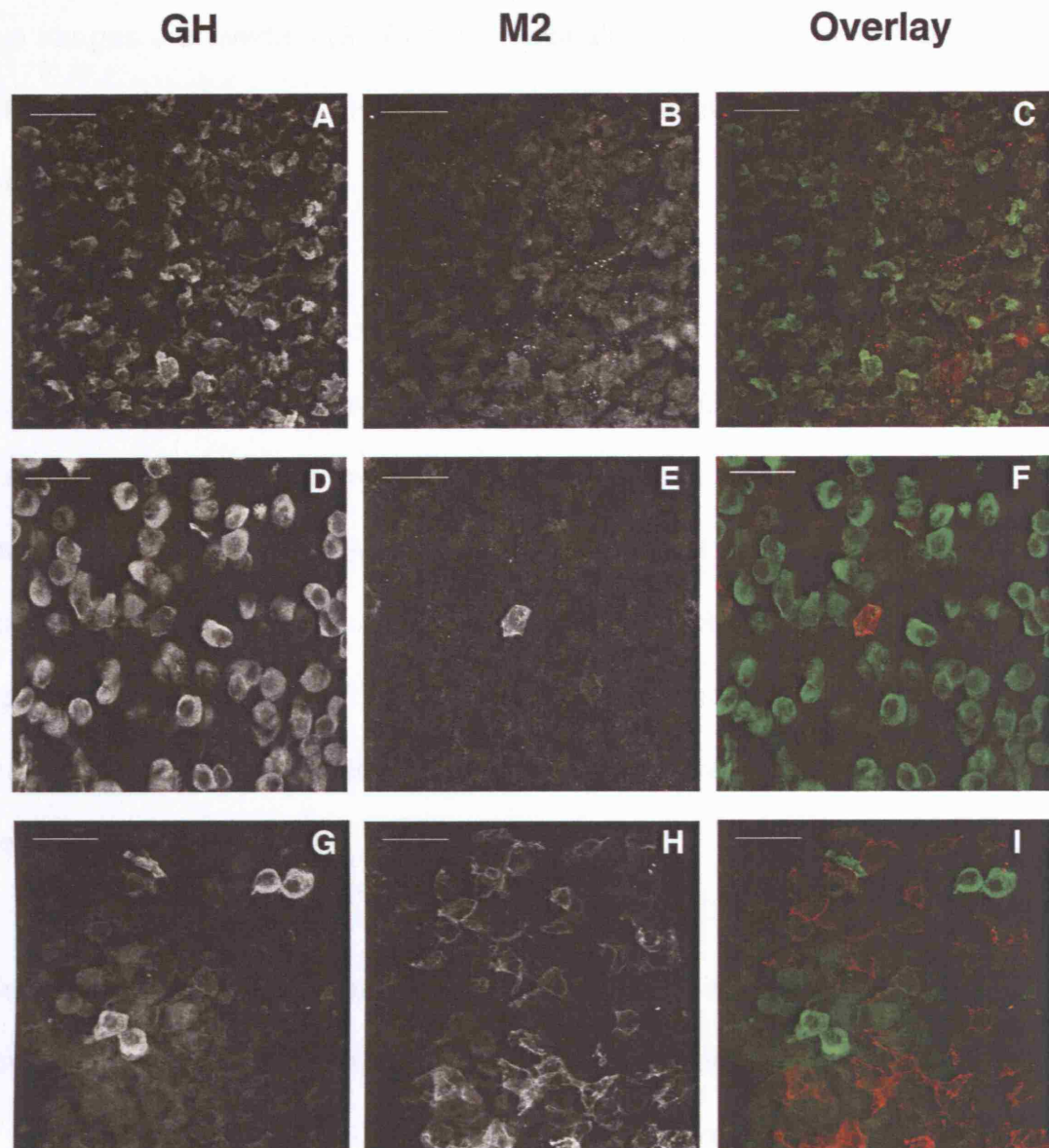


Figure 3.7 Immunohistochemistry for M2 on pituitary sections.

Immunofluorescence for GH (green) and M2 (red) on non-transgenic (A-C), low copy line (D-F) and medium copy line (G-I) pituitary sections. Images were taken using an inverted confocal microscope with a 40x objective.

Scale bar is equal to 10 μ m.

two images are overlaid (3.7F) some colocalisation can be detected between GH and M2 positive cells. However, not all GH positive cells appeared positive for M2.

In the medium copy line pituitary it was obvious that there were fewer GH positive cells than in the low copy line pituitary (3.7G). There was also some more diffuse staining with the GH antibody. Comparison to the other GH immunofluorescence (3.7A and D) would suggest that this is specific GH staining, although background staining cannot be ruled out. M2 positive cells can be detected (3.7H). These cells exhibit a generally membranous staining for M2. The majority of the remaining M2 positive cells appear to be GH negative (3.7I).

Colocalisation of GH and M2 is not seen in the majority of cells that are positive for M2 with the immunostaining, especially in the medium copy line (figure 3.7I). It is possible that the M2 positive cells are expressing GH but at levels undetectable with the antibody. Equally, it may be that the GH positive cells are expressing low levels of M2, which remain undetectable with this assay. The ability to detect colocalisation of GH and M2 will depend on the ability of cells to survive M2 channel activity and maintain GH synthesis and storage. Thus only the “surviving” cells may be selected in this manner.

Cells that are immunopositive for M2 in both the low and medium copy lines (figure 3.7 E and H) have an altered morphology. These cells no longer

resemble the spherical shape of a healthy GH cell and appear to have an irregular, extended shape.

3.2.7 Analysis of pituitary growth hormone (GH) levels

The pituitary GH content for the three lines was determined by RIA to assess the effect of GH-M2 expression on hormone levels. Pituitaries were dissected from low and medium copy line mice and their non-transgenic littermates at 14, 42 and 100 days of age. Pituitaries were obtained from high copy line mice at 42 days only, due to the low number of offspring from this line. The growth hormone profile for the low and medium copy lines was established by RIA and is shown in figure 3.8. Data is presented for 4-7 animals per group.

At 14 days male and female low and medium copy line mice (fig. 3.8 A and B, respectively) have significantly reduced GH contents compared to their non-transgenic littermates. Low copy male mice have a pituitary GH content that is 25% that of their non-transgenic littermates (T: $2.21\mu\text{g/pituitary} \pm 0.58$; NT: $8.82\mu\text{g/pit} \pm 0.98$; $P < 0.001$), whereas female mice have a GH content of $1.31\mu\text{g/pit} \pm 0.22$ ($P < 0.001$) compared to $10.12\mu\text{g/pit} \pm 1.45$ (13% of non-transgenic content). This level is further reduced in the medium copy line mice. Males have a GH content of $1.4\mu\text{g/pit} \pm 0.38$ (compared to $9.09\mu\text{g/ml} \pm 1.39$; 15%; $P < 0.01$) and females 0.41 (no SEM as only $n=1$ is above detectable level) compared to $12.3\mu\text{g/pit} \pm 2.3$ (3%).

In low copy line mice there has been a relative increase in the pituitary GH content by 42 days (figure 3.8C), although a deficit remains compared to non-transgenic littermates. Male mice have a GH content of $32.46\mu\text{g/pit} \pm 7.04$ ($P<0.01$) which is 54% of the non-transgenic littermate content ($59.93\mu\text{g/pit} \pm 4.0$) and female mice have a GH content of $30.77\mu\text{g/pit} \pm 5.06$ ($P<0.05$), which is 58% of the non-transgenic littermate GH content ($52.83\mu\text{g/pit} \pm 8.09$). This increase is less pronounced in medium copy line mice (figure 3.8D) where the male mouse GH content has risen to 25% of the control (T: $11.29\mu\text{g/pit} \pm 5.56$; NT: $45.05\mu\text{g/pit} \pm 3.91$; $P<0.01$) and the female GH content is only 7% of the non-transgenic level (T: $4.3\mu\text{g/pit} \pm 1.32$; NT: $57.78\mu\text{g/pit} \pm 18.89$; $P<0.05$). Figure 3.8E shows high copy line male and female GH contents at 42d. Transgenic mice of this line are severely affected and have a GH level that is below the level of detection of the assay (approximately $0.005\mu\text{g/pit}$). The data presented are from 3-5 animals.

At 100 days, the GH content of low copy line male and female mice is beginning to fall in relation to that observed for NT mice (figure 3.8F). The GH content of male mice is 46% that of the control (T: $25.18\mu\text{g/pit} \pm 4.7$ vs. NT: $54.34\mu\text{g/pit} \pm 5.11$; $P<0.001$) and in females the GH content is 55% (T: $26.41\mu\text{g/pit} \pm 2.63$ vs. NT: $47.65\mu\text{g/pit} \pm 11.04$; $P<0.05$). In medium copy line mice there has been a large relative decrease in the pituitary GH content compared to non-transgenic littermates (figure 3.8G). Male mice have a GH content of $2.62\mu\text{g/ml} \pm 1.06$ ($P<0.001$) which is 3% of the non-transgenic littermate content ($85.88\mu\text{g/pit} \pm$

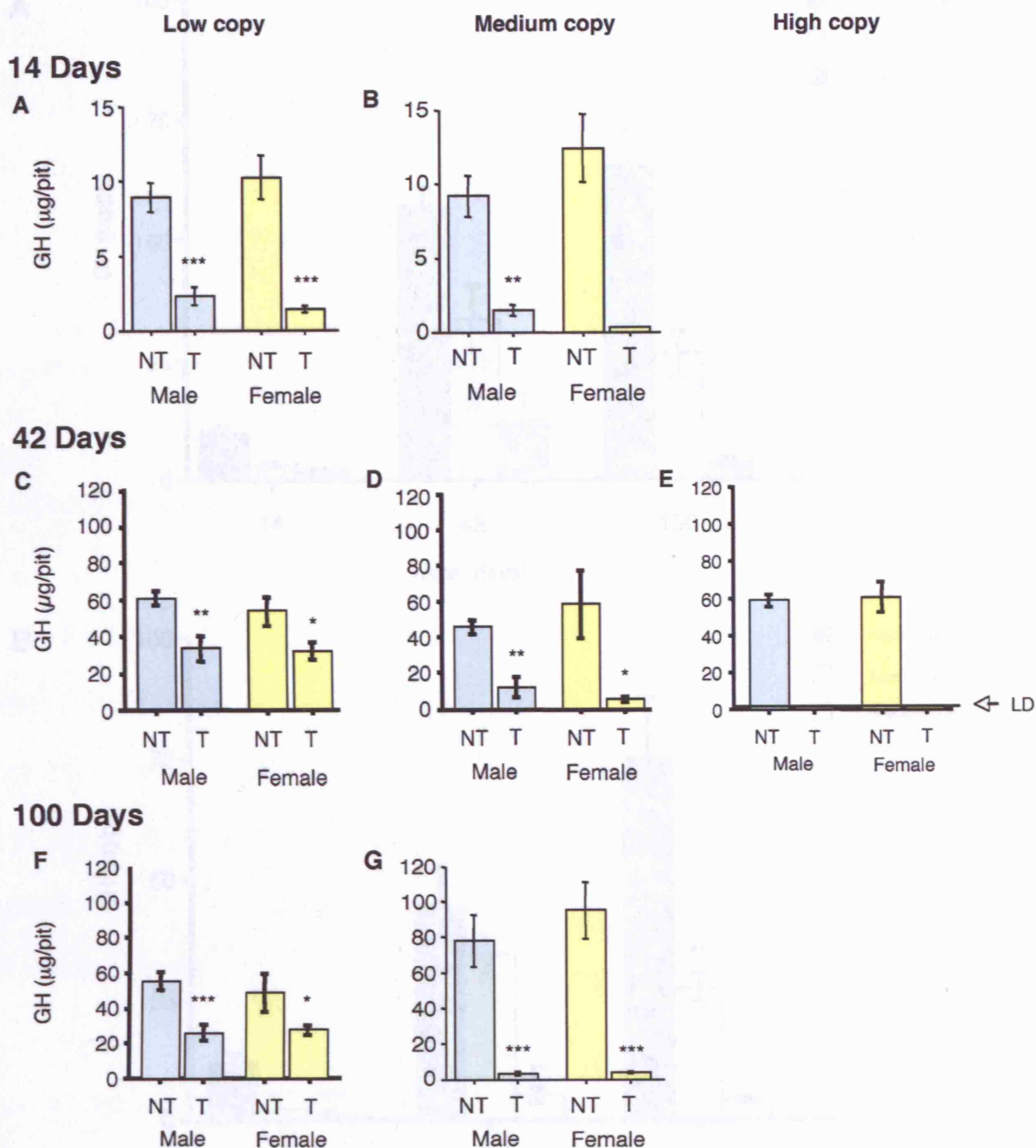


Figure 3.8 Pituitary growth hormone content of low, medium and high copy line mice

Pituitary GH content ($\mu\text{g/pit}$) was measured at 14, 42 and 100 days by mGH radioimmunoassay.

14 days - (A) low copy, (B) medium copy

42 days - (C) low copy, (D) medium copy, (E) high copy

100 days - (F) low copy, (G) medium copy

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. NT vs. T. Unpaired student t-test.

LD = level of detection and indicates the lowest measurable value using the mGH RIA (approx. $0.005\mu\text{g/pit}$).

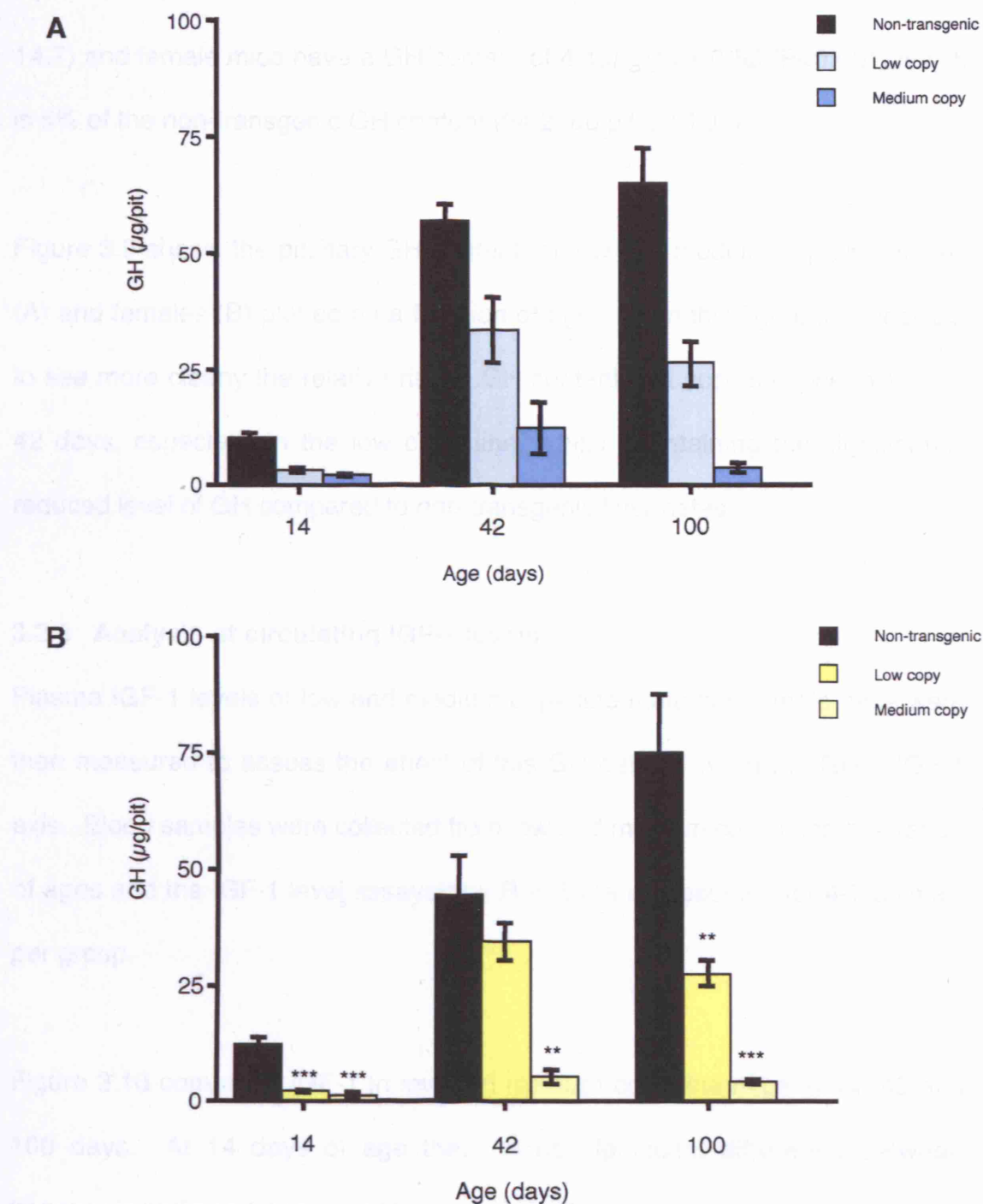


Figure 3.9 Pituitary GH content of low and medium copy line mice compared to non-transgenic littermates as a function of age

(A) Pituitary GH content ($\mu\text{g/pit}$) of male low and medium copy line mice compared to non-transgenic littermates at 14, 42 and 100 days.

(B) Pituitary GH content ($\mu\text{g/pit}$) of female low and medium copy line mice compared to non-transgenic littermates at 14, 42 and 100 days.

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. Transgenic vs non-transgenic. Unpaired student t-test.

14.7) and female mice have a GH content of $4.43\mu\text{g/pit} \pm 0.58$ ($P < 0.001$) which is 5% of the non-transgenic GH content ($94.24\mu\text{g/pit} \pm 16.03$).

Figure 3.9 shows the pituitary GH content for low and medium copy line males (A) and females (B) plotted as a function of age. From this figure it is possible to see more clearly the relative rise in GH content that occurs between 14 and 42 days, especially in the low copy line, whilst maintaining the significantly reduced level of GH compared to non-transgenic littermates.

3.2.8 Analysis of circulating IGF-1 levels

Plasma IGF-1 levels of low and medium copy line male and female mice were then measured to assess the effect of this GH deficiency on the GH – IGF-1 axis. Blood samples were collected from low and medium copy mice at a range of ages and the IGF-1 level assayed by RIA. Data is presented for 4-7 animals per group.

Figure 3.10 compares IGF-1 in low and medium copy line mice at 14, 42 and 100 days. At 14 days of age there is no significant difference between transgenic and non-transgenic littermates of either the low or medium copy lines (fig 3.10 A and B).

Neither the low nor the medium copy lines are significantly IGF-1 deficient at 42 days compared to control littermates (fig 3.10C and D, respectively). At 100 days, IGF-1 levels of low copy line mice are not significantly different to non-

transgenic values (fig 3.10F). Medium copy line mice appear to show a decrease in plasma IGF-1 (fig 3.10G) but this difference is not statistically significant.

IGF-1 levels were below the threshold for this assay in the plasma of high copy line male and female mice (fig 3.10E).

3.2.9 Rimantadine treatment

42-day low copy line male mice, and non-transgenic littermates, were treated with 1mg/ml Rimantadine in their drinking water for two weeks to test the reversibility of effects of expression of open M2 channel *in vivo*, as described previously (Le Tissier et al., 2005). At the end of this period pituitaries were dissected and assayed for GH content by RIA. Figure 3.11A shows the pituitary GH content of treated low copy line and non-transgenic mice compared to their untreated controls. There was no significant increase in treated mice compared to the untreated controls.

It is possible that delivery of Rimantadine in the drinking water does not provide the mice with a substantial or continuous enough dose to cause an effect. In order to try to overcome this, a second method of drug delivery was tested to see if the effect of the M2 channel could be blocked with a more consistent drug delivery method. Low copy line male mice, and non-transgenic littermates, at 30 days of age were used. Mini-pumps were inserted subcutaneously to release a continuous dose of Rimantadine over two weeks. At the end of the

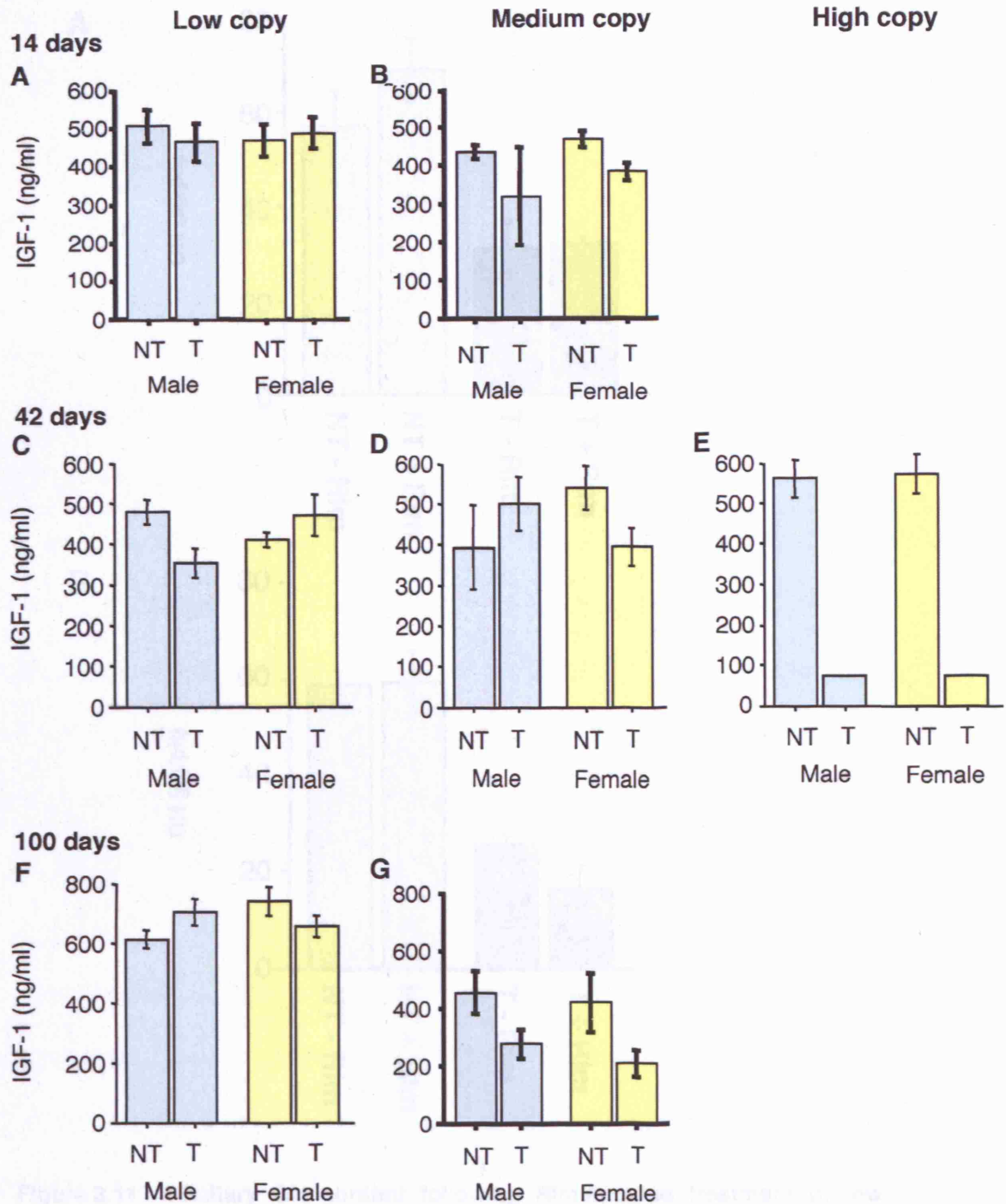


Figure 3.10 Plasma IGF-1 levels of male and female low and medium copy line mice

Plasma IGF-1 (ng/ml) was measured by RIA at 14, 42 and 100 days.

14 days - (A) low copy, (B) medium copy. 42 days - (C) low copy, (D) medium copy.

100 days - (E) low copy, (F) medium copy.

* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. Transgenic vs non-transgenic. Unpaired student t-test.

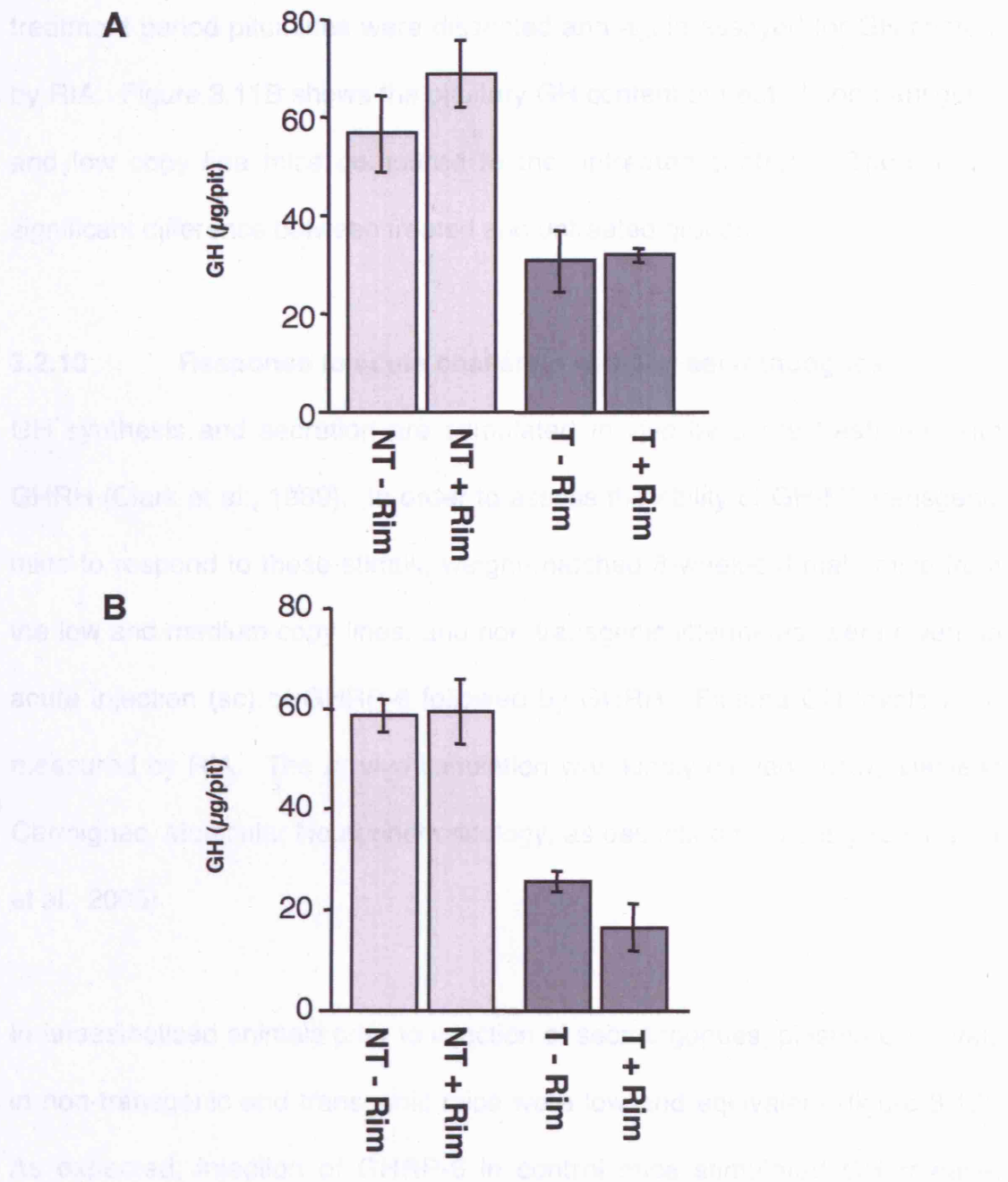


Figure 3.11 Pituitary GH content following Rimantadine treatment of low copy line male mice

(A) Transgenic and non-transgenic littermates were treated with 1mg/ml Rimantadine in drinking water for three weeks and pituitary growth hormone content measured by RIA.

(B) Mini-pumps containing 4.2mg Rimantadine were implanted subcutaneously. Transgenic and non-transgenic littermates were treated for two weeks and pituitary growth hormone content measured by RIA.

Unpaired student t-test (n=5-6). Treated vs untreated.

treatment period pituitaries were dissected and again assayed for GH content by RIA. Figure 3.11B shows the pituitary GH content of treated non-transgenic and low copy line mice compared to the untreated controls. There is no significant difference between treated and untreated groups.

3.2.10 Response to acute challenge with GH secretagogues

GH synthesis and secretion are stimulated *in vivo* by acute treatment with GHRH (Clark et al., 1989). In order to assess the ability of GH-M2 transgenic mice to respond to these stimuli, weight-matched 8-week-old male mice from the low and medium copy lines, and non-transgenic littermates, were given an acute injection (sc) of GHRP-6 followed by GHRH. Plasma GH levels were measured by RIA. The *in vivo* canulation was kindly carried out by Danielle Carmignac, Molecular Neuroendocrinology, as described previously (Le Tissier et al., 2005).

In anaesthetised animals prior to injection of secretagogues, plasma GH levels in non-transgenic and transgenic mice were low and equivalent (figure 3.12). As expected, injection of GHRP-6 in control mice stimulated GH release. However, the response in the low (LC) and medium copy (MC) line mice was much less than that measured for non-transgenic littermates (NT: 45.6ng/ml \pm 13.9 ($P<0.01$); LC: 10.25ng/ml \pm 1.86 ($P<0.01$); MC: 15.5ng/ml \pm 4.11 ($P<0.05$)). Plasma GH levels returned to pre-stimulation levels in both non-transgenic and transgenic groups. Upon stimulation with GHRH, an increase in plasma GH was again observed. In non-transgenic mice, the level of the resp-

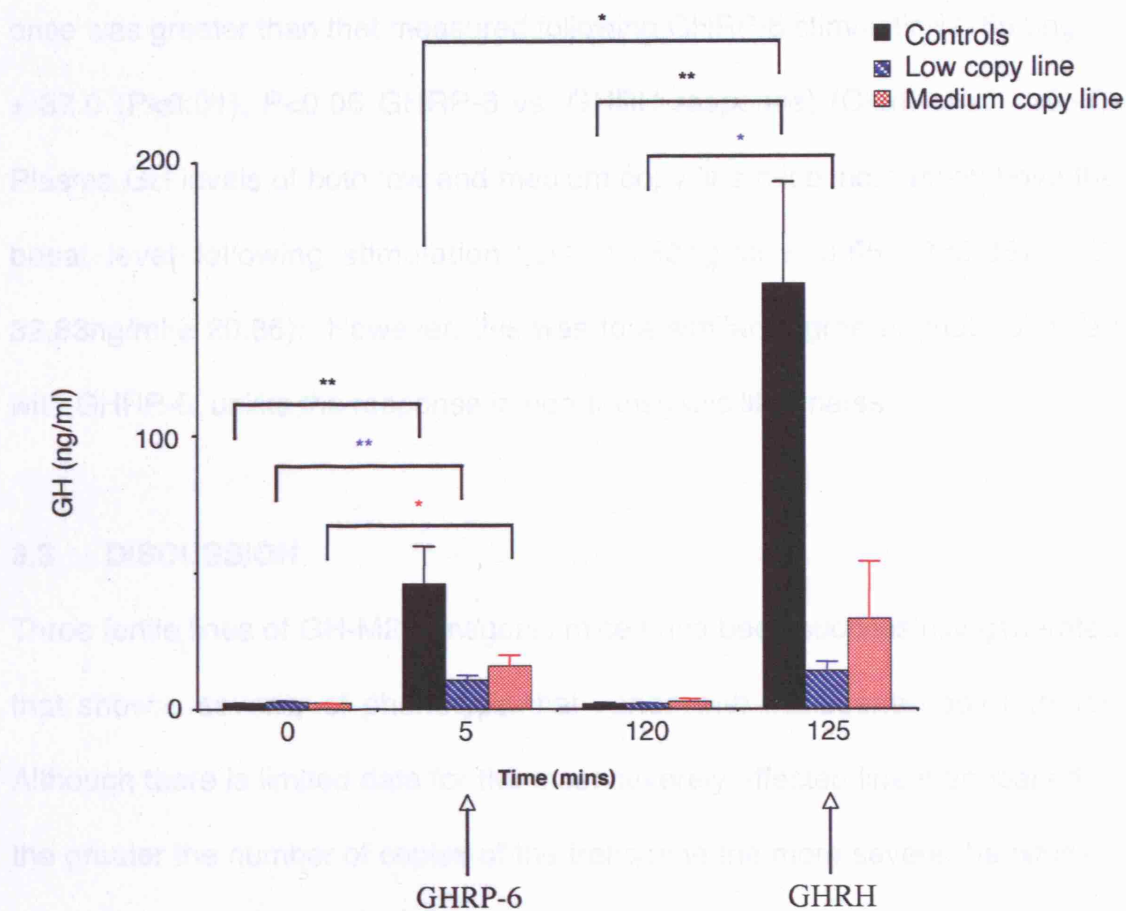


Figure 3.12 Plasma GH following acute challenge with GHRP-6 and GHRH in low copy, medium copy and non-transgenic littermates

Plasma GH (ng/ml) measured before and 5 minutes after injection of GHRP-6 (10 μ g/ml) and GHRH (2 μ g/ml) in non-transgenic (black), low copy line (blue) and medium copy (red) littermates at 8 weeks.

Unpaired student t-test (n=4-6). * P<0.05, ** P<0.01; non-transgenic littermates.

* P<0.05, ** P<0.01; low copy line. * P<0.05; medium copy line.

onse was greater than that measured following GHRP-6 stimulation (155.3ng/ml \pm 37.0 ($P<0.01$); $P<0.05$ GHRP-6 vs. GHRH response) (Clark et al., 1989). Plasma GH levels of both low and medium copy line mice increased above the basal level following stimulation (LC: 13.63ng/ml \pm 3.65 ($P<0.05$); MC: 32.83ng/ml \pm 20.86). However, this was to a similar degree as that observed with GHRP-6, unlike the response in non-transgenic littermates.

3.3 DISCUSSION

Three fertile lines of GH-M2 transgenic mice have been successfully generated that show a severity of phenotype that varies with transgene copy number. Although there is limited data for the most severely affected line it appears that the greater the number of copies of the transgene the more severe the pituitary effect appears to be.

Using reverse transcription PCR it has been possible to detect M2 expression in RNA isolated from the pituitary. Surprisingly, M2 expression was also detected in the kidney and testes of the medium copy line, but not low copy or non-transgenic, mice. This was also found to be the case for the GHRH-M2 model (Le Tissier et al., 2005). Other tissues were tested but found to be negative for both GH and M2. Two different primer sets were used to confirm the result and reactions set up without the reverse transcriptase as a negative control for contamination. Although use of the hGH LCR has been reported to be highly specific (Bennani-Baiti et al., 1998; Magoulas et al., 2000), it is possible that this ectopic expression is a result of the hGH LCR used and that this is not sufficient

for pituitary-specific expression of the transgene. This could be tested using immunohistochemistry to study GH and M2 expression in the kidney and testes. Luque *et al.* (2007) have also reported transgene expression in the testes, although a short promoter was used in this model. Despite detectable M2 transcripts amplified from the testes, it is likely that it is at a low level, or in a cell type not related to fertility, as low and medium copy line male mice are used to maintain the lines and fertility does not appear to be compromised. Litter sizes are also comparable to wild type pairings. The poor breeding of the high copy line is thought to be a result of the severe hormone deficiency induced by somatotroph ablation and can be improved by giving a course of twice-daily hGH injections from 3 weeks of age.

In order to image expression of the GH-M2 transgene product, immunofluorescence for GH and M2 was used. This technique is reliant on the presence of GH cells and will not detect those already ablated. It was not possible to detect a high level of colocalisation between GH and M2, suggesting that M2 and GH are expressed exclusively with no colocalisation. However, this is unlikely as M2 expression is driven by the GH LCR, targeting specific expression of the transgene to somatotrophs (Shewchuk *et al.*, 1999). It is expected that transgenic cells will still synthesise endogenous GH. Studies of the GH-GFP mouse that used the GH LCR to target GFP to somatotrophs (Magoulas *et al.*, 2000) revealed colocalisation of GFP and endogenous GH in the secretory granules. It is possible that expression of M2 adversely affects GH storage and secretion by disrupting golgi function (Henkel and Weisz, 1998;

Henkel et al., 2000). This in turn may reduce the amount of GH stored in secretory vesicles resulting in diffuse, cytoplasmic GH making detection of GH cells by immunostaining more difficult. On the other hand, in newly generated GH cells the level of M2 expressed at the plasma membrane may be too low to be detected by immunofluorescence with the polyclonal M2 antibody. GH cells that are immunonegative for M2 have a round morphology associated with normal GH cells (Magoulas et al., 2000). This may suggest a low level of M2 channel activity that has yet to disrupt cell function or to cause cell ablation. It may be possible to detect coexpression of GH and M2 using *in situ* hybridisation. This method is used to detect mRNA transcripts so may provide evidence for disruption of somatotroph function prior to ablation. For example, if M2 channel activity is disrupting the secretory pathway of GH it is possible that, although the protein may be undetectable with immunostaining techniques, GH synthesis is still occurring in these somatotrophs. However, the M2 probe used in these experiments gave a high level of background making it difficult to detect any positive staining. It is possible that development of a different M2 probe would increase specificity of binding.

The relationship between transgene copy number and phenotype was illustrated initially by comparison of the changes in body weight and length compared to Southern blot analysis for the three lines. The growth of the low copy line, with at least one copy of the transgene, appears to be unaffected compared to non-transgenic controls. The medium copy line has twice as many copies of the transgene. Gain in body weight of these mice occurs at a slower

rate than their non-transgenic littermates. At the onset of puberty these mice have a mild but significant delay in weight gain compared to controls but by 8 weeks of age, post-puberty, body weight appears to be normal. This suggests that the GH cell population is still responsive to GHRH, the main trigger for GH release, resulting in sufficient GH release to allow growth and development to catch up. IGF-1 levels are not significantly affected during this period, also indicating that GH secretion is sufficient to maintain IGF-1 secretion. The remaining GH positive cells may be secreting GH at a higher rate to make up for the suspected cell loss caused by M2 channel activity. This higher turnover of GH may contribute to the decrease in measurable pituitary GH content but a maintained IGF-1 level. However, male medium copy line mice have a shorter body length at 8 weeks suggesting a defect in longitudinal bone growth. Although, IGF-1 levels appear normal the pattern of stimulation of IGF-1 secretion by GH may be affected in these transgenic mice. M2 channel activity may alter the pulsatile pattern of GH secretion (Eden, 1979; Clark et al., 1986), for example by preventing the calcium channel activity required for secretion of GH (Chen et al., 2000). It is also possible that somatotroph ablation results in break down of the somatotroph network which is thought to aid coordinated secretion (Bonnetfont et al., 2005), which would in turn have downstream effects on growth. It would be interesting to sample the plasma GH and IGF-1 levels during puberty in the medium copy line mice. However, the plasma sampling technique used in rats (Clark et al., 1986) is difficult to perform in mice over long periods of time. Keith Fairhall, Molecular Neuroendocrinology, has developed a method for sampling from mice. This is a scaled-down method of that used for

sampling from rats. The length of sampling time is dependent on the size of the mouse. This may help to establish the reason for the delay and subsequent rebound in growth of this line of mice.

Studies of the diphtheria toxin mouse model (Behringer et al., 1988) revealed that GH was undetectable in the pituitary and plasma of transgenic mice, despite a small percentage (approx 0.01%) of somatotrophs remaining in these transgenic pituitaries. Plasma IGF-1 levels were subsequently reduced in these mice compared to controls. In the low and medium copy GH-M2 mice, however, GH is detectable by RIA and IGF-1 levels are not significantly affected in low copy line mice and medium copy line mice to 42 days of age, suggesting that the action of M2 may be progressive and activity does not immediately result in cell ablation.

In the low copy line mice, and to a lesser extent in the medium copy line mice, there is a relative increase in pituitary GH content by 42 days. This indicates that the GH cell population is still responding to the GHRH stimulus during puberty, with the ability to undergo proliferation and/or increase GH synthesis (Barinaga et al., 1983; Barinaga et al., 1985). It is unclear whether this increase is a result of stimulating the remaining GH cells to produce more GH or if it is production of new GH cells. If it was an effect on the remaining GH cells it may be expected that the increase in GH content would only be small and temporary, as these cells would already have been subjected to M2 channel activity. Further stimulation of GH production will also result in increased M2

expression and may lead to more rapid ablation of these cells. By producing a new population of GH cells it would be possible to have a longer term increase in pituitary GH content as it would take time for M2 to accumulate and cause an effect. Newer cells would therefore be able to function relatively normally in comparison to older cells in the population. The amount of, and length of exposure to, M2 channel activity that is required to kill a cell is not yet clear, however. It is possible that M2 channel activity is affecting the storage and secretion of GH prior to inducing cell death. An inability of the somatotrophs to secrete GH may result in an increase in detectable GH in the pituitary samples by RIA. The increase in pituitary GH observed between 14 and 42 days may therefore represent the progression of M2 channel expression and activity, although maintenance of plasma IGF-1 levels indicates that these mice can still secrete sufficient levels of GH.

Another possibility, however, is that not all the cells express the transgene and that it is in fact a mosaic of expression that enables the pituitary to continue producing a sufficient level of GH to maintain growth during puberty. It is also possible that, upon incorporation of the transgene into the genome, a portion of the LCR was lost following a DNA break or rearrangement, for example. Whilst PCR amplification has shown the presence of the LCR it is still unclear whether the LCR is appropriately positioned 5' of the transgene which could affect the specificity of transgene expression. Cells that do not express M2 could therefore survive and function normally enabling the plasma IGF-1 level, and therefore growth, to be maintained.

In an attempt to reverse the GH deficient phenotype induced by M2 channel activity, mice were treated with Rimantadine and the pituitary GH content measured following treatment compared to untreated controls. The apparent survival of a subpopulation of the somatotrophs and an increase in pituitary GH content at puberty in the presence of M2 suggested that the stem cell population may have remained intact, providing the possibility for reversal of the GH deficiency. However, treatment of the low copy line mice with Rimantadine has not resulted in rescue of the phenotype, indicating that expression of M2 may be irreversible *in vivo* or that the treatment is ineffective *in vivo* at the doses administered. The least affected line was chosen for this rescue experiment as it was assumed that due to the presence of some pituitary GH and a lesser amount of M2 this line is likely to have cells that are able to survive in the presence of M2. These cells may be more likely to undergo differentiation/proliferation when channel activity is blocked by Rimantadine treatment.

Two methods of drug delivery were tested. The first experiment was to give post-pubertal male mice Rimantadine in the drinking water. After 3 weeks of treatment there was no change in the pituitary GH content and it was still significantly decreased compared to non-transgenic littermates. This could be a result of the method of drug delivery. On average mice of 30g will drink approximately 5 ml of water a day. This means that the most Rimantadine that each mouse could be exposed to is 5 mg per day from the 1mg/ml dose. It was

not possible to increase the dose as previous work by Danielle Carmignac revealed that mice treated with a greater dose of Rimantadine became sick and began to lose weight during treatment. There are also limits on the solubility of Rimantadine in water. Treating mice in this way does not provide a continuous dose of the drug. Rimantadine will be metabolised by the liver and removed from the system of the mouse, with metabolites appearing 30 minutes after drug administration (Hoffman et al., 1988). We could not be sure that we could achieve a sufficient level of Rimantadine.

In order to try to overcome this, a continuous method of drug delivery was tested. Mini-pumps containing 4.2mg of Rimantadine were inserted subcutaneously to release 12.5 μ g of the drug hourly. After two weeks of treatment there was no change in the pituitary GH content compared to either the untreated controls or the non-transgenic littermates. The actual dose of Rimantadine received by the mouse over the treatment period was less than that received through the previous treatment despite the continuous nature of the treatment. This proved to be insufficient to block the effect of M2 expression. It was not possible to increase the amount of Rimantadine used in this experiment as licence procedure, size of mouse, volume of mini-pump and solubility of drug all prevent giving a more substantial mini-pump dose. This second method of treatment was delivered at 30 days, compared to 42 days for the first Rimantadine experiment. This was decided as a result of work recently published by Bonnefont *et al.*, 2005, which describes a dramatic increase in somatotroph clustering and network formation from 30 to 60 days of age. By

initiating the treatment of our mice at the start of this period, when the somatotroph proliferation drive may be greatest, it was hoped it would be possible to maximise the effect of the Rimantadine treatment.

Acute treatment with GHRH and the GH secretagogue GHRP-6 is known to stimulate GH secretion in rodents (Clark and Robinson, 1985a; Clark et al., 1989). Blood samples were taken from anaesthetised low and medium copy line male mice before and after acute stimulation with GHRP-6 and GHRH. It was expected that a graded response would be observed in line with copy number and pituitary GH content. However, the rise in plasma GH levels of the low and medium copy lines was similar after stimulation and significantly decreased compared to non-transgenic littermates. The reduced response of the transgenic mice is likely to be a result of M2 channel activity affecting the secretory response as well as reducing the amount of GH available for release. Stimulation of somatotrophs by GHRH results in activation of voltage-gated calcium channels through the cAMP pathway (Mayo, 1992; Gaylinn et al., 1993), whereas GHRP-6 stimulation activates IP_3 resulting in release of calcium from intracellular stores (Cheng et al., 1991; Lei et al., 1995; Mau et al., 1995). The presence of open M2 channels in the cellular membranes is likely to alter the ion gradient across the membrane as sodium and potassium ions will be able to flow freely across the membranes along their ion gradient. This in turn will alter the membrane potential and prevent the activation of the calcium channels that is required for secretion to occur. For example, M2 channel

activity would prevent depolarisation of the plasma membrane and subsequent activation of the voltage-gated calcium channels.

The similarity of the response of the low and medium copy line males suggests that low copy expression of the transgene is sufficient to affect the somatotroph response to GHRH and GHRP-6. It may also be that the remaining GH cells respond at their maximum capacity to endogenous GHRH, which may lead to desensitisation of the GHRH receptor (Kovacs et al., 1997). This would also act to decrease GH secretion in response to acute GHRH stimulation.

To summarise, three GH-M2 lines have been generated by pronuclear microinjection. There is a variable GH deficient and growth phenotype across these lines that is copy number dependent. IGF-1 levels are not significantly affected in the low and medium copy lines to 100 days of age. It has not been possible to reverse the GH deficiency in low copy line mice with Rimantadine treatment, possibly due to the methods of drug delivery chosen and accessibility of the drug to the pituitary.

4. ANALYSIS OF SECONDARY EFFECTS OF GH-M2 EXPRESSION AND HORMONAL MANIPULATIONS

4.1 INTRODUCTION

In the previous chapter, the effect of GH-M2 expression on GH cells was assessed. I have shown that the severity of the GH deficient phenotype is dependent on transgene copy number and that despite the decrease in pituitary GH levels, circulating IGF-1 in low and medium copy lines was unaffected.

Previous models of somatotroph ablation have illustrated that lactotrophs were also affected by targeting GH cells, indicating that these two cell types are produced from a common precursor cell during pituitary development. For example, in the model developed by Borrelli *et al.* (1989) thymidine kinase ablation of GH cells resulted in a marked decrease in PRL cell number. Upon recovery from this process GH cell number increased followed by an increase in the number of PRL cells after a prolonged period of recovery. Immunohistochemistry revealed no significant changes to thyrotroph, corticotroph and gonadotroph populations. Behringer *et al.* (1988) showed a similar, non-reversible effect upon somatotroph ablation with diphtheria toxin. A reduction in the number of lactotrophs was observed, although this was to a lesser degree than that of somatotrophs. Effects on thyrotroph, gonadotroph or corticotroph cells following GH ablation were not investigated. Li *et al.* (1990) have shown that the Snell dwarf mouse (Pit-1 mutant) does not generate somatotrophs, lactotrophs or thyrotrophs during development. The Ames dwarf mouse carries a mutation in *Prop1* (Sornson *et al.*, 1996) that prevents

expansion of the endocrine cell lineages during development (Gage et al., 1996a). These mice have decreased pituitary gonadotropin contents as well as decreased amounts of pituitary GH, PRL and TSH. Little mice have a reduced pituitary GH content as a result of a mutation in the GHRH receptor (Godfrey et al., 1993) that prevents a response to GHRH (Clark and Robinson, 1985b). A PRL deficiency is also observed in these mice (Eicher and Beamer, 1976). In contrast to these mouse models, the dwarf rat (dw/dw) has an increased number of PRL cells that coincides with the somatotroph hypoplasia caused by the dwarf mutation (Tierney and Robinson, 2002). Other mouse models such as the autosomal dominant GH deficient mouse (McGuinness et al., 2003) and the antigen-activated T cell model (de Jersey et al., 2002) result in variable pituitary hormone deficiencies that are a result of less direct factors such as macrophage-induced bystander killing and antigen dosage, respectively. It is therefore of interest to consider the impact of somatotroph ablation on other pituitary cell types.

This chapter addresses the effect of GH-M2 expression on lactotrophs, thyrotrophs and gonadotrophs to assess the impact of GH-M2 channel activity on the pituitary. The effect of estrogen treatment on low and medium copy line male mice has also been assessed. Estrogen is known to stimulate PRL synthesis in lactotrophs (Shull and Gorski, 1984) and somatotrophs (Goth et al., 1996) as well as stimulating lactotroph proliferation (Hentges et al., 2000; Chaturvedi and Sarkar, 2005) and to some extent GH synthesis and secretion (Gonzalez-Parra et al., 1996).

4.2 RESULTS

4.2.1 Analysis of pituitary prolactin (PRL) content

In many GH-deficient transgenic mouse models an effect is also observed in the lactotroph population. Following assessment of the variable GH deficiency in the three GH-M2 lines it was important to analyse the effect of this on pituitary prolactin content. The pituitary prolactin (PRL) profile for the three GH-M2 mouse lines was established by RIA and is shown in figure 4.1. Pituitary extracts previously tested for GH content at 14, 42 and 100 days were used (n=4-7).

The non-transgenic pituitary PRL content at 14 days is less than $1\mu\text{g/pit}$ in both males and females (M: $0.24\mu\text{g/pit} \pm 0.06$; F: $0.36\mu\text{g/pit} \pm 0.06$). The PRL content of the male pituitary increases gradually with age to $3.02\mu\text{g/pit} \pm 0.2$ by 100 days. In contrast, female non-transgenic mice have a more dramatic increase in pituitary PRL content by 42 days ($5.65\mu\text{g/pit}$) which more than doubles by 100 days ($12.16\mu\text{g/pit} \pm 1.25$).

High copy line (figure 4.1E) male mice have a significantly reduced pituitary PRL content at 42 days of age ($0.03\mu\text{g/pit} \pm 0.01$ vs. $1.17\mu\text{g/pit} \pm 0.11$; $P < 0.001$; T vs. NT). Although the female value is decreased this is not statistically significant, possibly due to the large standard error of the non-transgenic value ($0.39\mu\text{g/pit} \pm 0.05$ vs. $3.17\mu\text{g/pit} \pm 0.87$; T vs. NT).

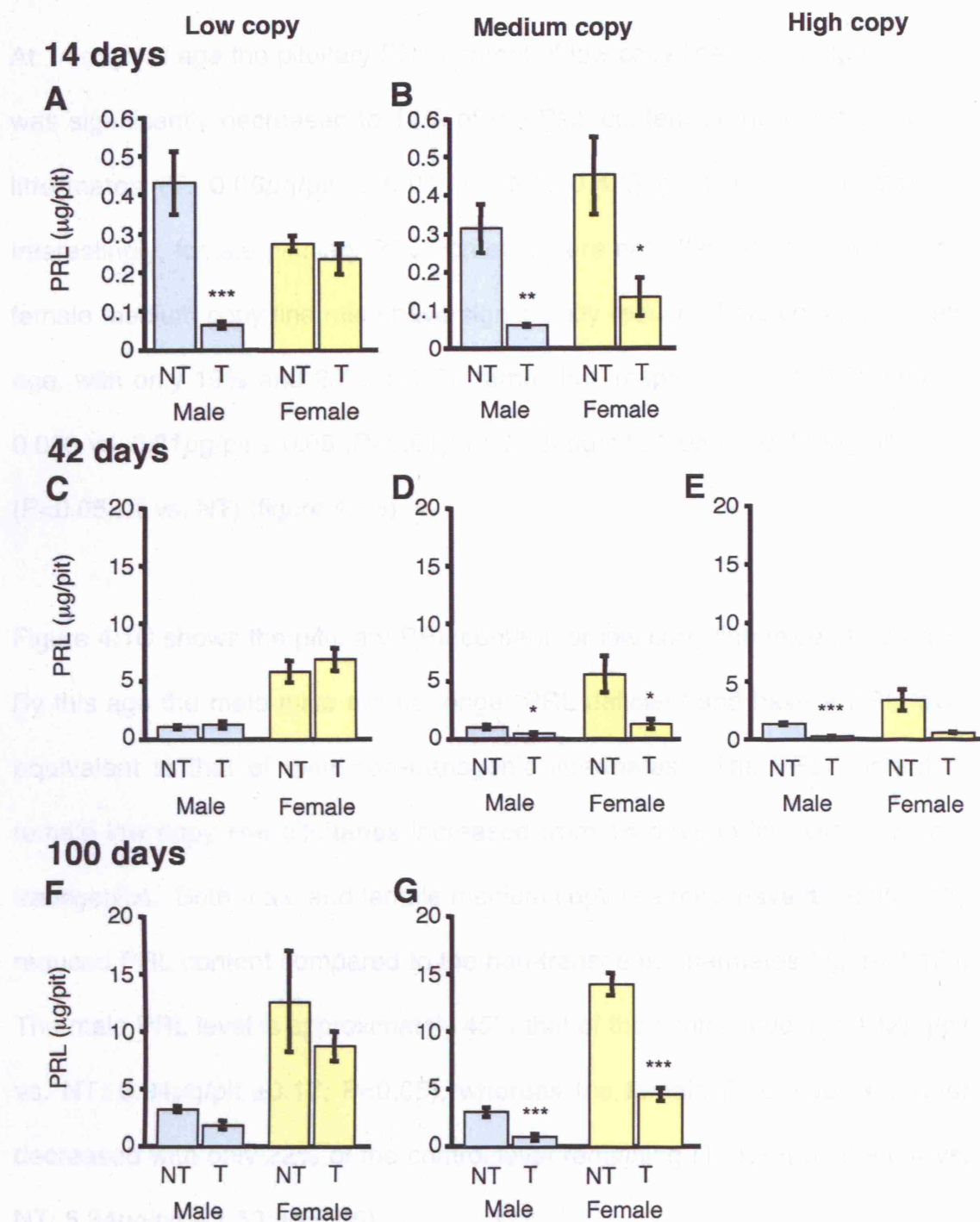


Figure 4.1 Pituitary PRL content of low and medium copy line mice at 14, 42 and 100 days and high copy line mice at 42 days.

PRL levels ($\mu\text{g/pituitary}$) were measured in male and female mice by RIA and compared to non-transgenic littermates.

14 days – low copy (A) and medium copy line (B), 42 days – low copy (C), medium copy (D) and high copy line (E), 100 days – low copy (F) and medium copy line (G).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. T vs. NT as measured by unpaired Student t-test.

At 14 days of age the pituitary PRL content of low copy line males (figure 4.1A) was significantly decreased to 14% of the PRL content of the non-transgenic littermates (T: $0.06\mu\text{g/pit} \pm 0.01$ vs. NT: $0.425\mu\text{g/pit} \pm 0.08$; $P<0.001$). Interestingly, female pituitary PRL contents were not affected. Both male and female medium copy line mice have significantly reduced PRL contents at this age, with only 19% and 29% of PRL remaining, respectively (M: $0.06\mu\text{g/pit} \pm 0.003$ vs. $0.31\mu\text{g/pit} \pm 0.06$ ($P<0.01$); F: $0.132\mu\text{g/pit} \pm 0.05$ vs. $0.448\mu\text{g/pit} \pm 0.1$ ($P<0.05$); T vs. NT) (figure 4.1B).

Figure 4.1C shows the pituitary PRL content for low copy line mice at 42 days. By this age the male mice are no longer PRL deficient and have a PRL level equivalent to that of their non-transgenic littermates. The PRL content of female low copy line pituitaries increased from 14 days in line with the non-transgenics. Both male and female medium copy line mice have a significantly reduced PRL content compared to the non-transgenic littermates (figure 4.1D). The male PRL level is approximately 45% that of the control level (T: $0.42\mu\text{g/pit}$ vs. NT: $0.94\mu\text{g/pit} \pm 0.17$; $P<0.05$), whereas the female PRL level is further decreased with only 22% of the control level remaining (T: $1.18\mu\text{g/pit} \pm 0.4$ vs. NT: $5.34\mu\text{g/pit} \pm 1.53$; $P<0.05$).

At 100 days the low copy line pituitary PRL of both male and female mice remains at a level similar to the non-transgenic littermates (figure 4.1F). The decrease of PRL content of medium copy line male mice (figure 4.1G) is more pronounced at 100 days as the fold increase of the non-transgenic PRL level

from 42 days is greater than that observed in transgenic mice (T: $0.7\mu\text{g/pit} \pm 0.27$ vs. NT: $2.9\mu\text{g/pit} \pm 0.41$; $P < 0.001$). The fold increase observed in the amount of pituitary PRL in medium copy line females (3.75 fold) is greater than that of non-transgenic littermates (2.6 fold), however there is still a significant difference in PRL content (T: $4.43\mu\text{g/pit} \pm 0.58$ vs. NT: $13.98\mu\text{g/pit} \pm 0.98$; $P < 0.001$).

4.2.2 Analysis of pituitary thyroid stimulating hormone (TSH)

Thyrotrophs differentiate from a Pit-1 expressing precursor cell but are thought to form a separate lineage from somatotrophs and lactotrophs. The pituitary TSH content of low, medium and high copy line mice was assayed to analyse the impact of GH-M2 expression on this cell type. As for the PRL analysis, pituitary extracts were tested for their TSH content by RIA and the results are shown in figure 4.2.

Non-transgenic and low copy line male and female pituitary TSH contents are similar at the ages studied. At 14 days the non-transgenic TSH content is $0.35\mu\text{g/pit} \pm 0.07$ and $0.22\mu\text{g/pit} \pm 0.06$ for males and females, respectively. In low copy line mice pituitary TSH content was $0.34\mu\text{g/pit} \pm 0.08$ in males and $0.28\mu\text{g/pit} \pm 0.05$ in females (figure 4.2A). By 42 days of age (figure 4.2C) a small increase has occurred in the non-transgenic male pituitary TSH content, which is mirrored in the low copy line male pituitary. The non-transgenic and low copy line female pituitary TSH contents do not show this increase. At 100 days of age the male non-transgenic pituitary TSH content is $0.83\mu\text{g/pit} \pm 0.08$

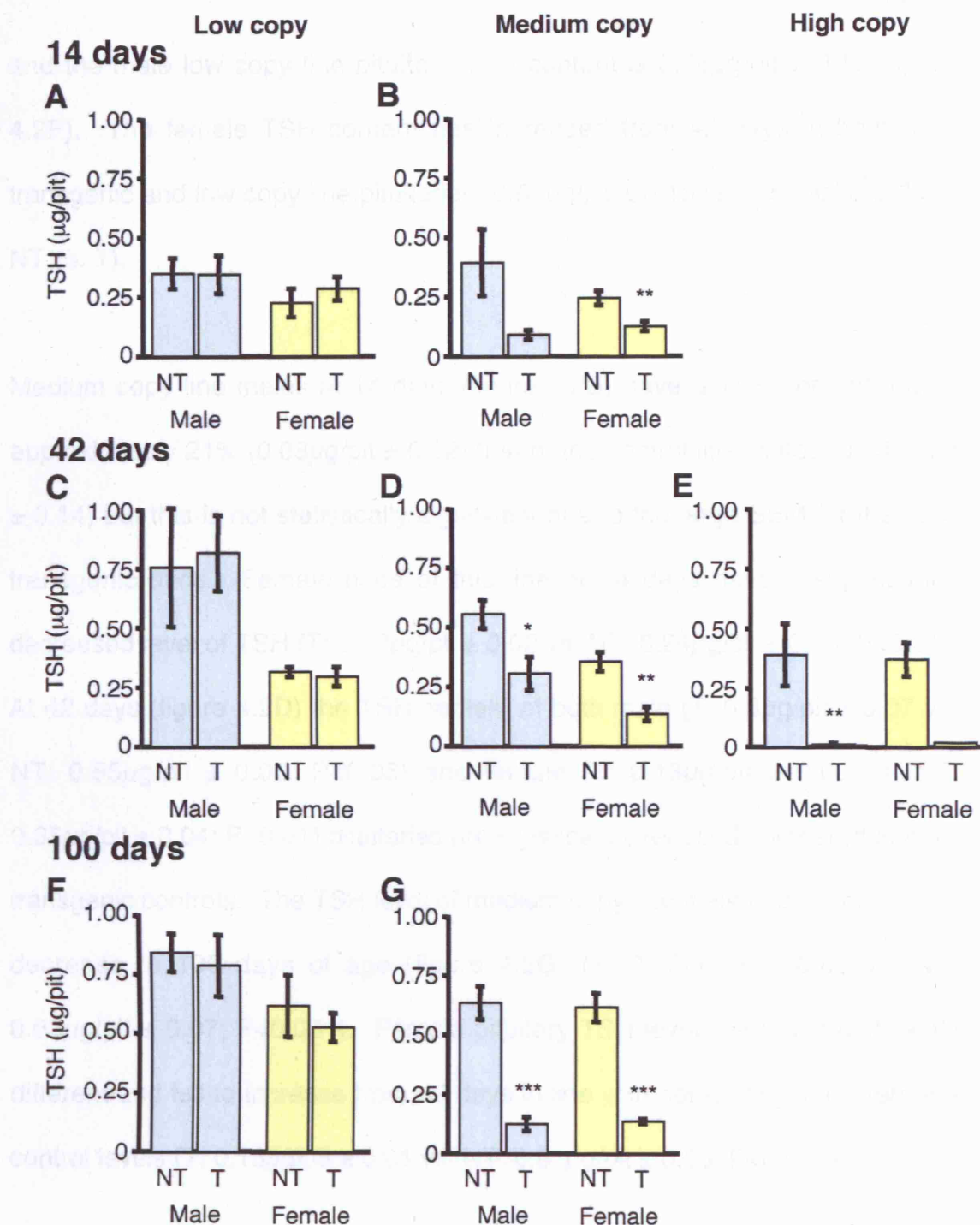


Figure 4.2 Pituitary TSH content of low and medium copy line mice at 14, 42 and 100 days and high copy line mice at 42 days.

TSH levels ($\mu\text{g/pituitary}$) were measured in male and female mice by RIA and compared to non-transgenic littermates.

14 days – low copy (A) and medium copy line (B), 42 days – low copy (C), medium copy (D) and high copy line (E), 100 days – low copy (F) and medium copy line (G).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. T vs. NT as measured by unpaired Student t-test.

and the male low copy line pituitary TSH content is $0.78\mu\text{g/pit} \pm 0.13$ (figure 4.2F). The female TSH content has increased from 42 days in both non-transgenic and low copy line pituitaries ($0.61\mu\text{g/pit} \pm 0.13$ vs. $0.52\mu\text{g/pit} \pm 0.06$; NT vs. T).

Medium copy line males at 14 days (figure 4.2B) have a TSH content that is approximately 21% ($0.08\mu\text{g/pit} \pm 0.02$) that of the control littermates ($0.39\mu\text{g/pit} \pm 0.14$) but this is not statistically significant due to the large SEM for the non-transgenic mice. Female mice of this line at 14 days have a significantly decreased level of TSH (T: $0.12\mu\text{g/pit} \pm 0.02$ vs. NT: $0.24\mu\text{g/pit} \pm 0.03$; $P < 0.01$). At 42 days (figure 4.2D) the TSH content of both male (T: $0.3\mu\text{g/pit} \pm 0.07$ vs. NT: $0.55\mu\text{g/pit} \pm 0.06$; $P < 0.05$) and female (T: $0.13\mu\text{g/pit} \pm 0.03$ vs. NT: $0.35\mu\text{g/pit} \pm 0.04$; $P < 0.01$) pituitaries are significantly reduced compared to non-transgenic controls. The TSH level of medium copy line male mice continues to decrease to 100 days of age (figure 4.2G) (T: $0.12\mu\text{g/pit} \pm 0.03$ vs. NT: $0.63\mu\text{g/pit} \pm 0.07$; $P < 0.001$). Female pituitary TSH levels remain significantly different and fail to increase from 42 days in line with non-transgenic littermate control levels (T: $0.13\mu\text{g/pit} \pm 0.01$ vs. NT: $0.61\mu\text{g/pit} \pm 0.06$; $P < 0.001$).

High copy line pituitaries were assayed at 42 days only (figure 4.2E). The pituitary TSH content of high copy line males was significantly reduced compared to non-transgenic littermates (T: $0.004\mu\text{g/pit} \pm 0.0005$; NT: $0.38\mu\text{g/pit} \pm 0.13$; $P < 0.01$). In females a large difference was observed between transgenic and non-transgenic littermates (T: $0.001\mu\text{g/pit} \pm 0$; NT: $0.36\mu\text{g/pit} \pm$

0.07). However, this was not statistically significant as the transgenic mice (n=2) had identical TSH contents.

It has been difficult to find references to the changes that occur in mouse pituitary TSH content as measured by RIA to validate the values obtained. The values for older mice do appear to correlate to those observed by McGuinness *et al.* (2003).

4.2.3 Plasma T4 analysis

A decreased pituitary TSH content leads to a decrease in circulating thyroid hormone levels (T4 and T3) (Harris et al., 1978b). This in turn could lead to a decrease in GH synthesis and secretion. It has been shown, by studies of hypothyroidism (Burstein et al., 1979; Chernausek et al., 1983; Ramos et al., 1998) and physiological changes following thyroidectomy (Katakami et al., 1986; Downs et al., 1990), that a decrease in thyroid hormone results in decreased GH synthesis and secretion (Evans et al., 1982). In order to check whether the decreased pituitary TSH content measured in medium copy line mice could be a contributing factor to the significantly decreased pituitary GH content, plasma T4 levels were measured by RIA.

T4 levels remained unaffected in both male and female low and medium copy line mice at 42 and 100 days, as shown in figure 4.4. Non-transgenic plasma T4 levels vary from 44.19ng/ml \pm 2.56 for males and 43.37ng/ml \pm 8.55 for females at 42 days to 42.06ng/ml \pm 3.6 and 35.73ng/ml \pm 3.5, for males and

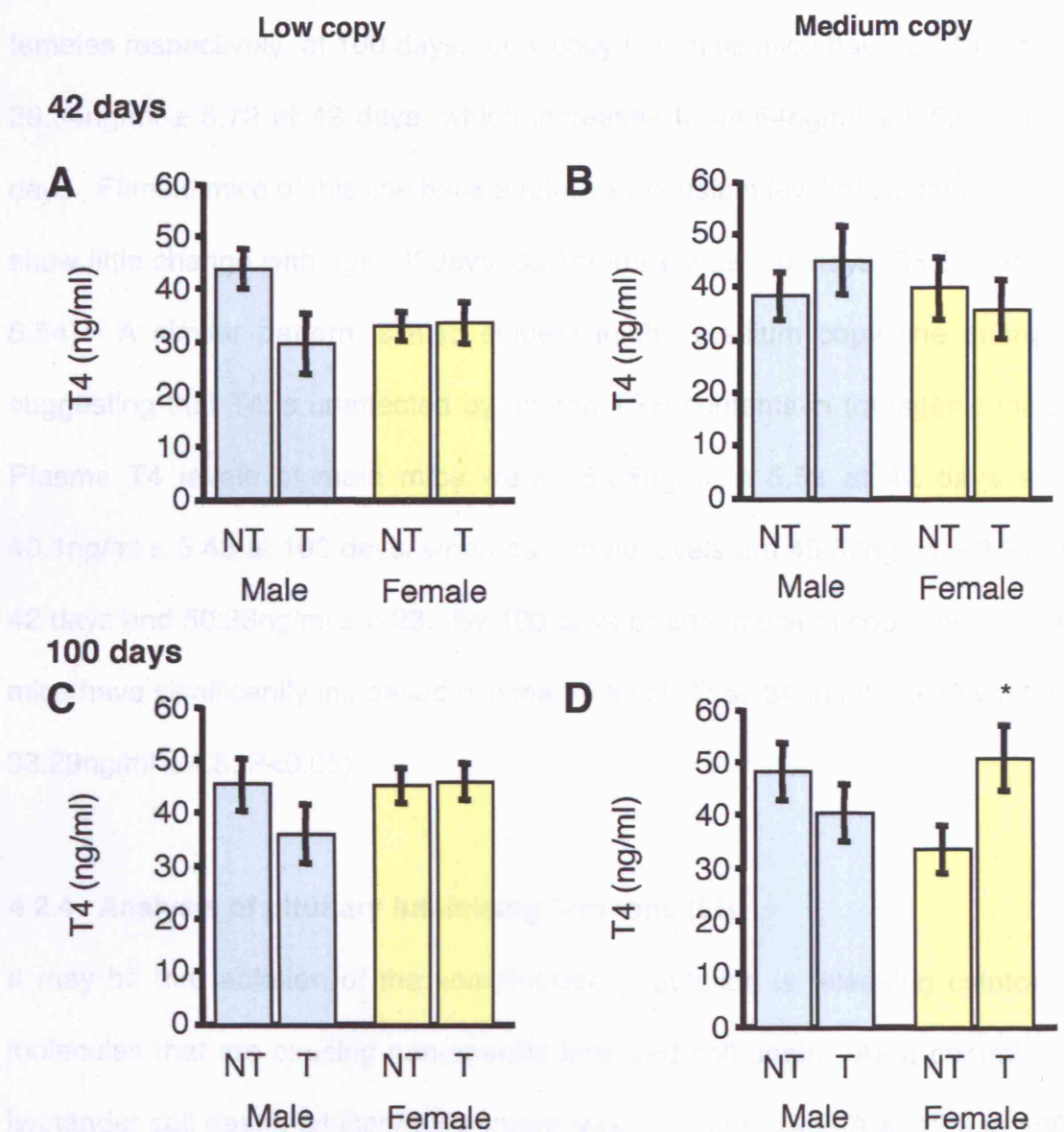


Figure 4.3 Low and medium copy line plasma T4 levels at 42 and 100 days.

Plasma T4 levels (ng/ml) were measured in male and female mice by RIA in low copy (A), medium copy (B) line mice at 42 days and in low copy (C) and medium copy (D) line mice at 100 days. * P<0.05. NT vs. T as measured by unpaired Student t-test.

females respectively, at 100 days. Low copy line male mice have a T4 level of 29.34ng/ml \pm 5.72 at 42 days, which increases to 44.64ng/ml \pm 6.52 by 100 days. Female mice of this line have a relatively constant level of plasma T4 and show little change with age (42days: 33.1ng/ml \pm 3.99; 100days: 35.28ng/ml \pm 5.54). A similar pattern is also evident in the medium copy line animals suggesting that T4 is unaffected by altered TSH contents in transgenic mice. Plasma T4 levels of male mice were 35.68ng/ml \pm 5.58 at 42 days and 40.1ng/ml \pm 5.43 at 100 days, whereas female levels are 45.57ng/ml \pm 3.34 at 42 days and 50.38ng/ml \pm 6.23. By 100 days of age, medium copy line female mice have significantly increased plasma T4 level (T: 50.34ng/ml \pm 6.23 vs. NT: 33.29ng/ml \pm 4.5; $P < 0.05$).

4.2.4 Analysis of pituitary luteinising hormone (LH)

It may be that ablation of the somatotroph population is releasing cytotoxic molecules that are causing non-specific localised cell death. As a control for bystander cell death, pituitary LH content was measured by RIA at 42 and 100 days in low and medium copy line mice, and at 42 days in high copy line mice, in order to assess the global impact on the pituitary of GH cell ablation. Gonadotrophs form a separate cell lineage to the Pit-1 lineage during pituitary development (Japon et al., 1994) and so should be unaffected by GH-M2 expression.

A sexual dimorphism in pituitary LH contents exists, with higher values recorded from the male pituitary of mice and rats (Watanabe, 1986). The male non-

transgenic value appears to increase slightly with age although not significantly. Female non-transgenic pituitary LH contents vary with the estrous cycle but do not appear to increase between 42 and 100 days. The LH levels recorded in male and female low copy line mice at 42 days (figure 4.4A) and 100 days (figure 4.4D) were not significantly different to those of their non-transgenic littermates. Medium copy line males showed no change at these two ages compared to non-transgenic control littermates (figures 4.4B and D, respectively). Female mice of this line, however, had a LH content equivalent to control littermates at 42 days (figure 4.4B) but at 100 days there was a significant difference between transgenic and non-transgenic littermates (NT: 545.60ng/pit \pm 64.71; T: 204.87ng/pit \pm 64.51; $P < 0.01$; figure 4.4E). This may be a result of the high non-transgenic value obtained. This is 3.6 fold higher than the value obtained for non-transgenic female littermates of the low copy line.

High copy line male mice have a significantly reduced pituitary LH content at 42 days (NT: 1606ng/pit \pm 295.2; T: 86.67ng/pit \pm 9.06; $P < 0.01$) as shown in figure 4.4C. Female high copy line mice have a reduced LH content although this is not statistically significant (NT: 152.2ng/pit \pm 59.35; T: 66.6ng/pit \pm 14.06). There is no longer a male-female difference in LH content in the high copy line mice.

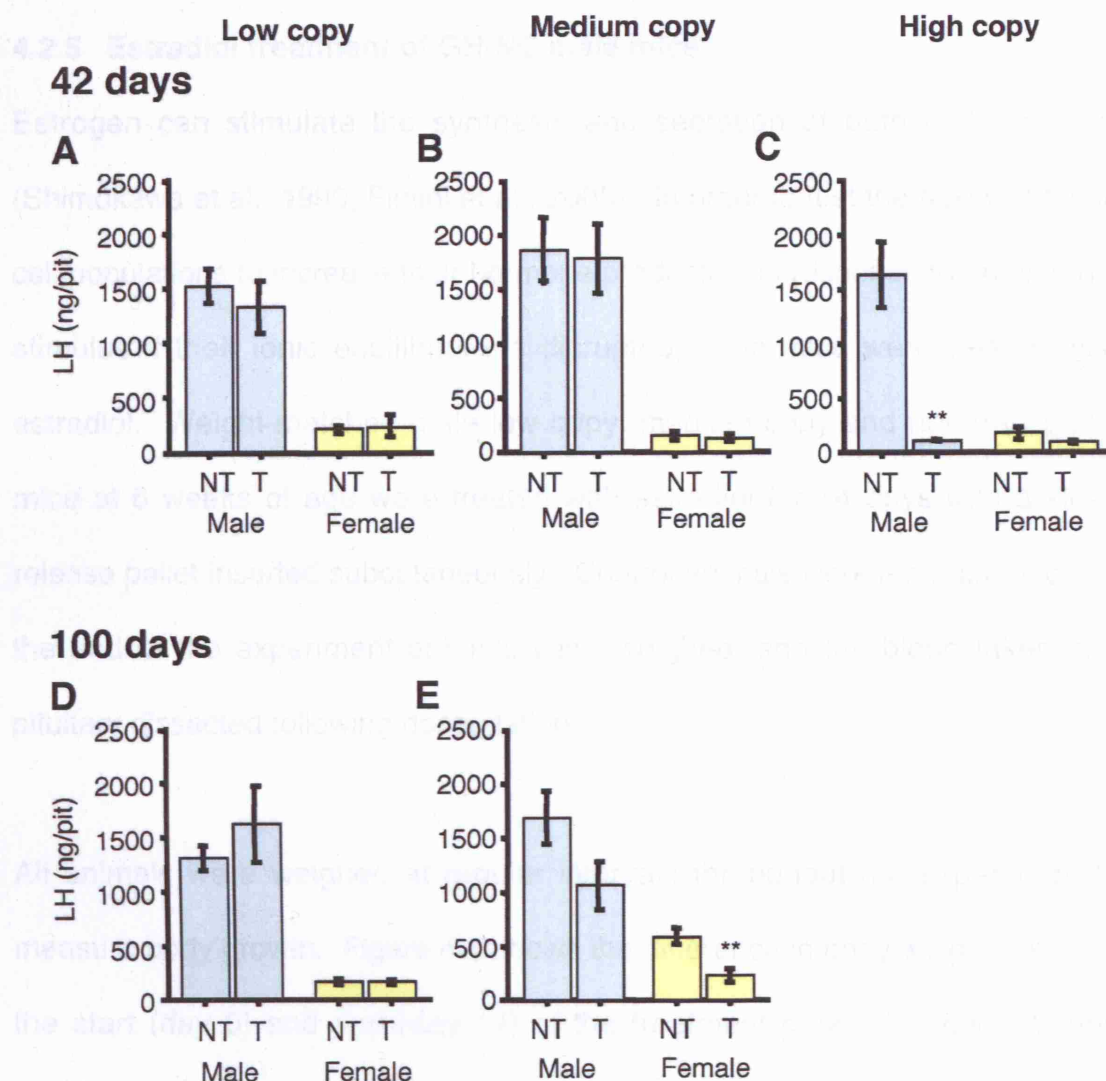


Figure 4.4 Pituitary LH content of low, medium and high copy line mice.

LH levels (ng/pituitary) were measured in male and female mice by RIA in low copy (A), medium copy (B) and high copy (C) line mice at 42 days and in low copy (D) and medium copy (E) line mice at 100 days. ** $P < 0.01$. NT vs. T as measured by unpaired Student t-test.

4.2.5 Estradiol treatment of GH-M2 male mice

Estrogen can stimulate the synthesis and secretion of both PRL and GH (Shimokawa et al., 1990; Fintini et al., 2005). In order to test the ability of these cell populations to increase their hormone production in response to an external stimulus if their ionic equilibrium is disrupted, male mice were treated with estradiol. Weight-matched male low copy, medium copy and non-transgenic mice at 6 weeks of age were treated with estradiol for 14 days with a slow-release pellet inserted subcutaneously. Control animals were left untreated. At the end of the experiment animals were weighed, and the blood taken and pituitary dissected following decapitation.

All animals were weighed at regular intervals throughout the experiment to measure body growth. Figure 4.5 shows the difference in body weight between the start (day 0) and end (day 14) of the treatment period for low (A) and medium (B) copy line males compared to untreated and non-transgenic controls. During the two-week period of this experiment the body weight of both treated and untreated mice increased as expected. This was to a lesser degree, however, in both transgenic and non-transgenic treated animals possibly due to an effect of treatment on food intake (Goth et al., 1996).

RIA was used to measure pituitary contents of GH, PRL and TSH of all of the experimental animals. An increase in both GH (figure 4.6A) and PRL (figure 4.6B) contents was found in low copy line male mice when treated with estradiol compared to untreated non-transgenic controls. The GH content of treated

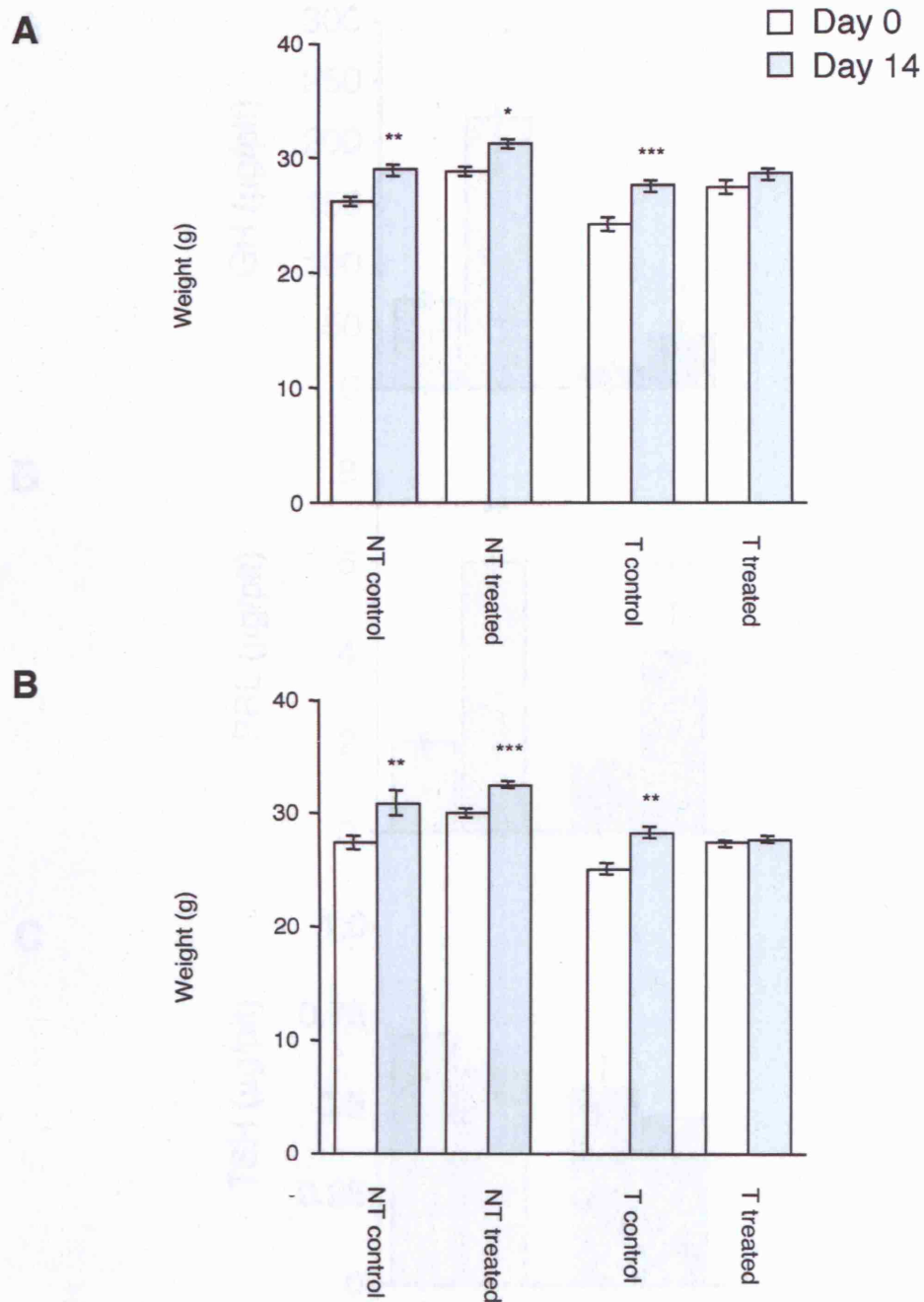


Figure 4.5 Body weights of experimental low and medium copy line mice after treatment with estradiol

Body weights (g) were measured before during and after the 14 day treatment with estradiol for treated and untreated animals. Body weights before and after treatment are plotted for low copy line (A) and medium copy line (B) animals. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. T vs. NT as measured by unpaired Student t-test.

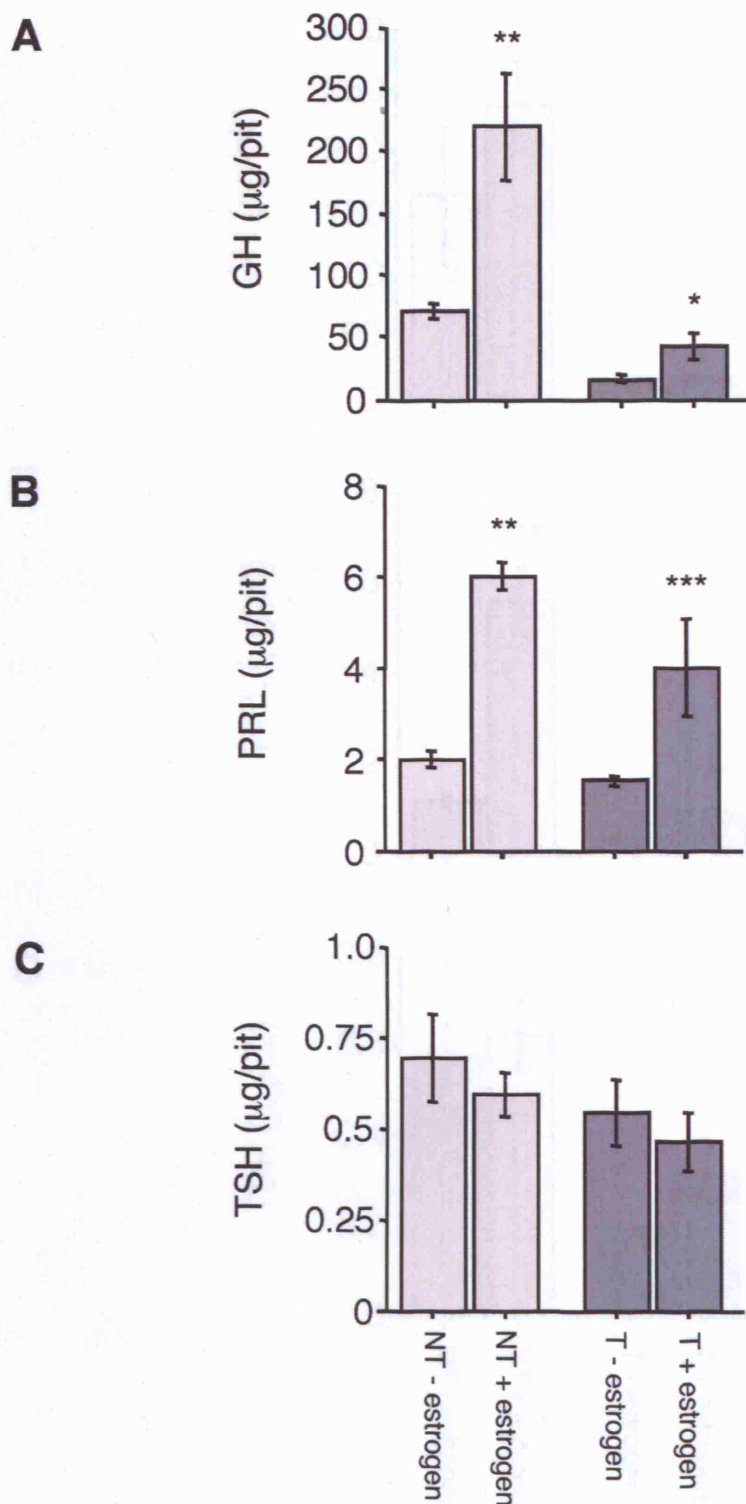


Figure 4.6 Pituitary hormone content of low copy line male mice following estradiol treatment.

Low copy line pituitary hormone contents were measured after two weeks of estradiol treatment by RIA for GH (A), PRL (B) and TSH (C). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. T vs. NT as measured by unpaired Student t-test.

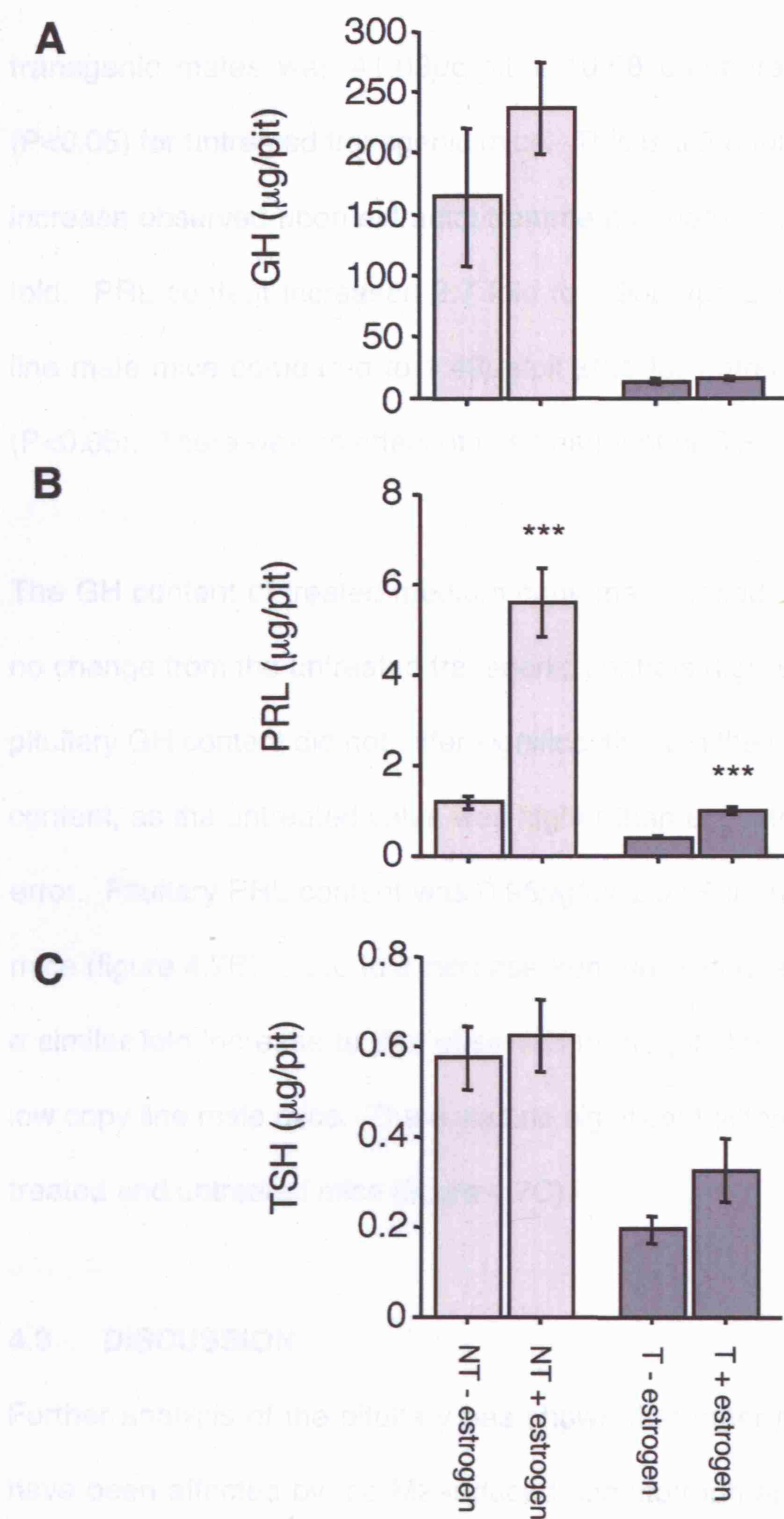


Figure 4.7 Pituitary hormone content of medium copy line male mice following estradiol treatment.

Medium copy line pituitary hormone contents were measured by RIA for GH (A), PRL (B) and TSH (C) after two weeks of estradiol treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. T vs. NT as measured by unpaired Student t-test.

transgenic males was $41.03\mu\text{g/pit} \pm 10.68$ compared to $14.47\mu\text{g/pit} \pm 2.3$ ($P<0.05$) for untreated transgenic mice. This is a 2.8 fold increase, whereas the increase observed upon estradiol treatment of non-transgenic littermates is 3.1 fold. PRL content increased 2.7 fold to $3.95\mu\text{g/pit} \pm 1.06$ in treated low copy line male mice compared to $1.49\mu\text{g/pit} \pm 0.1$ for untreated transgenic controls ($P<0.05$). There was no effect of this treatment on TSH levels (figure 4.6C).

The GH content of treated medium copy males failed to increase and showed no change from the untreated transgenic controls (figure 4.7A). Non-transgenic pituitary GH content did not differ significantly from the untreated non-transgenic content, as the untreated value was higher than expected with a large standard error. Pituitary PRL content was $0.95\mu\text{g/pit} \pm 0.09$ in treated medium copy line mice (figure 4.7B); a 2.6 fold increase from untreated levels ($P<0.001$). This is a similar fold increase to that observed in the pituitary PRL content of treated low copy line male mice. There was no significant difference in TSH contents of treated and untreated mice (figure 4.7C).

4.3 DISCUSSION

Further analysis of the pituitary has shown that other hormones, besides GH, have been affected by the M2-induced somatotroph ablation. It appears that, as with the variable GH deficiency, the effects on PRL, TSH and LH also vary in impact between the three GH-M2 lines suggesting they may also reflect the difference in transgene copy number and effect of the M2 product.

In low copy line mice PRL, TSH and LH pituitary contents are unaffected by M2 activity in the somatotrophs. The pituitary PRL content increases in line with non-transgenic values by 42 days of age although the subsequent increase observed in non-transgenic mice to 100 days appears affected in this transgenic line. Medium copy line mice have significantly reduced pituitary PRL and TSH contents from 14 days of age. An increase in PRL content is observed with age, particularly in females, although this is to a lesser extent than in the low copy line and values still remain significantly decreased compared to non-transgenic littermates. The most severe phenotype is observed in the high copy line mice with PRL, TSH and LH significantly decreased at 42 days of age.

Previous somatotroph ablation (Behringer et al., 1988; Borrelli et al., 1989) and dwarf (Roux et al., 1982; Tierney and Robinson, 2002) models have implicated lactotrophs as the major cell type altered by removal of the GH cell population. PRL and GH cells are thought to be generated by differentiation from a common Pit-1 containing precursor cell. It is therefore not unexpected that pituitary PRL content is also affected in the GH-M2 model. It was surprising, however, to find a difference in the pituitary TSH contents of these mice. Such a change was not reported in the thymidine kinase and diphtheria toxin ablation models, although in other models of induced GH deficiency a secondary effect on the thyrotroph population has been indicated (de Jersey et al., 2002; McGuinness et al., 2003). Thyrotrophs are believed to form a third, Pit-1 dependent lineage (Li et al., 1990; Simmons et al., 1990).

I considered several possibilities for why this might occur. The first is that new somatotrophs, generated from the response of progenitor cells to growth stimuli during puberty, can survive low level GH-M2 expression, as seen in the low copy line. However, as the copy number of the transgene increases the ability of the newly produced GH cells to survive is decreased. It may be that differentiation of the progenitor/precursor cells is driven towards replacing GH cells to account for the dramatic loss in cell number thus depleting the PRL and TSH cell populations, particularly in aging mice as the GH deficiency becomes more pronounced. In turn this may decrease the measurable pituitary PRL and TSH contents in comparison to non-transgenic littermates.

Secondly, it is possible that, due to the presence of Pit-1 binding sites in the hGH LCR (Bennani-Baiti et al., 1998), a low level of transgene expression is initiated in the progenitor or precursor cells of the Pit-1 lineage. Although transgene expression will be extinguished in differentiated PRL and TSH cells, a low level of M2 channel activity may persist in newly differentiated cells. This may slowly be removed from these cells through membrane recycling and regeneration. The M2 channel activity may affect the efficient storage and secretion of PRL and TSH. As the transgene copy number increases an increase in M2 expression in the progenitor cell and subsequent increase in residual expression and activity of M2 in the differentiated cells may occur. This may be sufficient to significantly alter the detectable pituitary PRL and TSH contents.

Three possible roles have been suggested for mammosomatotrophs: one, that they are a population of cells with the ability to express GH and PRL; two, that they are an intermediate/transition cell type in a somatotroph-lactotroph transdifferentiation pathway; and three, that they are a precursor cell for both the somatotroph and lactotroph populations. Mammosomatotrophs are predicted to make up 8-15% of the pituitary cell population (Frawley et al., 1985) although FACS analysis of rat pituitaries suggests this is a high estimate and in fact ranges from 2-8% depending on age and sex of the animal (Tierney and Robinson, 2002). Cells expressing both GH and PRL mRNA have been detected (Li et al., 1993) in pituitary adenomas. It is likely that this pool of mammosomatotroph cells is affected by GH-M2 expression. However, ablation of this small population of cells is unlikely to be the major cause for PRL deficiency in the medium and high copy lines if mammosomatotrophs form a distinct cell population. Alternatively, depletion of the somatotroph population by M2 channel activity may increase the drive for transdifferentiation of lactotrophs to somatotrophs, in turn decreasing the number of lactotrophs in the transgenic pituitary and the pituitary PRL content. Finally, if mammosomatotrophs are the precursor cell to somatotrophs and lactotrophs then ablation of this cell population would significantly affect the number of somatotrophs and lactotrophs produced. This precursor cell may be relatively unaffected in the low copy line but as M2 expression increases with transgene copy number an effect may become more apparent. This, however, does not account for the TSH deficiency of the medium and high copy lines.

The GH network, as illustrated by Bonnefont *et al.* (2005), exists throughout the anterior pituitary. If this is destroyed when the somatotrophs are ablated it is possible that it will have a knock on effect on the PRL cell population. If many PRL cells are produced secondary to GH cells (Hoeffler *et al.*, 1985) it may be that the two networks are closely related. By adversely affecting one a similar effect may be mirrored in the other. It has been suggested that thyrotrophs extend in a finger-like pattern from the ventral to dorsal surface of the anterior pituitary (Chauvet and Mollard, personal communication). This may also require an intact somatotroph network for formation.

Finally, it is possible that the PRL and TSH deficiency measured is a result of misexpression of the GH-M2 transgene. However, previous studies with the hGH LCR (Jones *et al.*, 1995; Magoulas *et al.*, 2000; Ho *et al.*, 2002) suggest that this is unlikely to occur in all of the lines.

In order to check that the GH deficiency was not enhanced by a thyroid hormone deficit as a result of reduced pituitary TSH contents, a total T4 assay was used to check thyroid function. T4 and T3 are produced by the thyroid and cause a wide range of effects, for example, on cell metabolism (Bernal and Refetoff, 1977) and development of the central nervous system (Pasquini and Adamo, 1994). A T4 deficiency would indicate that the mice are suffering from hypothyroidism (Surks, 1981; Liewendahl, 1983), possibly as a result of reduced pituitary TSH contents. Analysis of total T4 in the circulation of low and

medium copy line mice revealed no significant decrease in T4 levels compared to non-transgenic littermates at 42 and 100 days. The values obtained are comparable to wildtype plasma T4 levels previously published (Palha et al., 1994; Friedrichsen et al., 2003; Rabeler et al., 2004). The reduction in pituitary TSH content detected in medium copy line mice is therefore insufficient to adversely affect thyroid hormone production and secretion and in turn pituitary GH content. It is possible that aged medium (and high) copy line mice would become T4 deficient in line with a prolonged TSH deficiency, or that the turnover of pituitary TSH would be higher to maintain thyroid hormone levels.

Pituitary LH content was analysed in the low and medium copy GH-M2 lines at 42 and 100 days and high copy line mice at 42 days. The aim of this was to determine if the multiple hormone deficiency could be a result of apoptotic release of cytokines from affected GH cells in turn triggering local, non-specific cell death or if it is a result of M2 expression in other pituitary cell types. LH was unaffected in the pituitaries of low copy line mice, showing no difference to non-transgenic littermates. In medium copy line mice, however, a decrease in LH content was detected at 100 days in both males and females (figure 4.4E). This decrease was significant in females, but this was largely due to the unexpectedly high non-transgenic LH values in this group. Further repeats of this experiment are required to confirm this result, and indeed to check that the values obtained for the non-transgenic female littermates of the low copy line are not unusually low. If the difference in LH contents is truly significant at this late age, however, it indicates that it is not a result of non-specific M2

expression. If this cell type had been targeted with M2 then it would be expected that a deficiency would be detected at an earlier age as with GH, PRL and TSH. It is possible therefore that a decrease in pituitary hormones with age is a secondary result of GH cell death. A close proximity to the GH cells would also put them in the proximity of cytokines released from invading macrophages as observed in the autosomal dominant GH deficient (McGuinness et al., 2003) and GH immunoablation (de Jersey et al., 2002) mouse models. If this is the case, testing pituitaries from older low copy line mice may reveal a similar trend. In order to address this issue further work will include performing assays for apoptosis and necrosis on pituitaries from GH-M2 mice over a range of ages. For example, apoptosis can be detected by TUNEL staining, whereas it is possible to image necrosis by immunohistochemistry against specific macrophage markers or by studying incorporation of propidium iodide into dying cells. When performed with immunohistochemistry for GH it may be possible to image the scale of the effect of GH cell ablation.

Estrogen regulates prolactin gene expression by activating gene transcription (Lieberman et al., 1981; Gonzalez-Parra et al., 1996). It increases PRL synthesis in a number of ways including decreasing the number of dopamine receptors (Raymond et al., 1978) and increasing responsiveness to TRH (Giguere et al., 1982). The amount of dopamine released from the hypothalamus is reduced following long term treatment with estradiol (Cramer et al., 1979), increasing the synthesis and secretion of PRL. Estrogen treatment has been shown to induce the inhibitory effect of SST on lactotrophs in culture

(Kimura et al., 1986; Goth et al., 1996) which may increase the detectable PRL content in the pituitary following treatment.

In order to assess the responsiveness of the lactotroph, and somatotroph, population to the estrogen stimulus low and medium copy line male mice were treated with estrogen for 14 days. Low copy line male mice treated with estradiol had an increased pituitary GH and PRL content after treatment. In medium copy line mice, however, only the PRL content was increased with treatment. There was no effect on TSH contents in any of the treated groups. In both low and medium copy line mice pituitary PRL responds to estrogen treatment suggesting that the PRL cells are not themselves expressing M2, and that the decreased PRL levels detected in untreated mice is a secondary effect of targeting M2 to somatotrophs.

The relative recovery of pituitary growth hormone content of low copy line mice suggests that GH cells have the ability to survive in the presence of a low expression of M2. It is possible that these cells are newly produced and as a result have a relatively low level of M2 at the plasma membrane, which is not sufficient to yet cause cell ablation. 17β -estradiol has been shown to reduce the inhibitory effect of somatostatin on GH cells in primary cultures of rat anterior pituitaries (Gonzalez-Parra et al., 1996). Stimulation by GHRH is apparently unaffected by this treatment. This process will also be triggered in treated medium copy line mice, but it is possible that the higher level of M2 transcription prevents somatotroph survival. Other effects of estrogen treatment

include a reduction of the circulating level of IGF-1 in mice (Fintini et al., 2005), feminisation of the secretory profile of male rats (Painson et al., 1992) and reduction of food intake in rats (Goth et al., 1996). This may help to explain the difference in growth in treated and untreated mice.

In conclusion, in this chapter I have shown that the effect of GH-M2 expression on hormone cell types, secondary to somatotrophs, is transgene copy number dependent. It is unclear at this stage if this is an indirect effect of somatotroph ablation on the Pit-1 lineage or a bystander effect causing localised, non-specific cell death. This will be discussed further in Chapter 7.

5. IMAGING THE EFFECT OF M2 EXPRESSION ON PITUITARY CELL NETWORKS

5.1 INTRODUCTION

Initial confocal imaging studies of the GH-eGFP mouse (Magoulas et al., 2000) revealed that GH cells exist in large clusters. More recently, it has been shown that these clusters of somatotrophs form a complete and functional cell network throughout the adult male anterior pituitary, maintained by adherens junction formation (Bonnetfont et al., 2005). This network develops from the point of appearance of the first GH cells at e15.5 through to adulthood with an increase in clustering observed in young adult male pituitaries. These cells respond to GHRH in a coordinated manner as assessed by sequential Ca^{2+} imaging of clusters of GH cells. Imaging of the Prl-DsRed mouse, where lactotrophs are fluorescently tagged with DsRed, suggests that PRL cells may form a similar cell network within the anterior pituitary with close interactions with the somatotroph network.

I have previously described the primary and secondary pituitary phenotypes of the three GH-M2 transgenic mouse lines, as assessed by biochemical techniques. M2 expression in these transgenic mice appears to affect many parameters, most notably on cell viability and proliferation, in turn affecting endocrine cell number and distribution. This chapter aims to image the impact of GH-M2 transgene expression on the somatotroph and lactotroph populations and the structure of the anterior pituitary by immunofluorescence of fixed pituitary sections and 2-photon microscopy of whole fixed pituitaries. Low and

medium copy line mice have been crossed to GH-GFP and Prl-DsRed mice, individually, to aid imaging of these cell populations. This work has been done in collaboration with Patrice Mollard and his group at the Institut de Génomique Fonctionnelle, Montpellier.

5.2 RESULTS

5.2.1 Immunofluorescence of GH-M2 pituitaries

Immunofluorescence for GH, PRL and TSH was used to image, with confocal microscopy in 2D, the extent of these hormone deficiencies previously assessed in chapters 3 and 4. Sections of male pituitaries from the low and medium copy lines, and a female pituitary from the high copy line, at 8 weeks were imaged using age-matched male non-transgenic pituitary sections as a control. DAPI (blue) was used as to counterstain nuclei to provide a reference of total cell number. All sections compared are from a similar, central point through the pituitary.

Figure 5.1 shows large scale views of the staining obtained with the GH antibody in low (A) and medium (B) male and high (C) copy line female pituitaries. A varying degree of anterior pituitary hypoplasia is evident between the three lines and appears to correlate to transgene copy number and severity of GH deficiency. It is most apparent when compared to the non-transgenic pituitary (right-hand panels). The density of GH cells appears to decrease as the copy number increases, compared to the non-transgenic control. At this age the non-transgenic female pituitary has a large number of GH cells at a

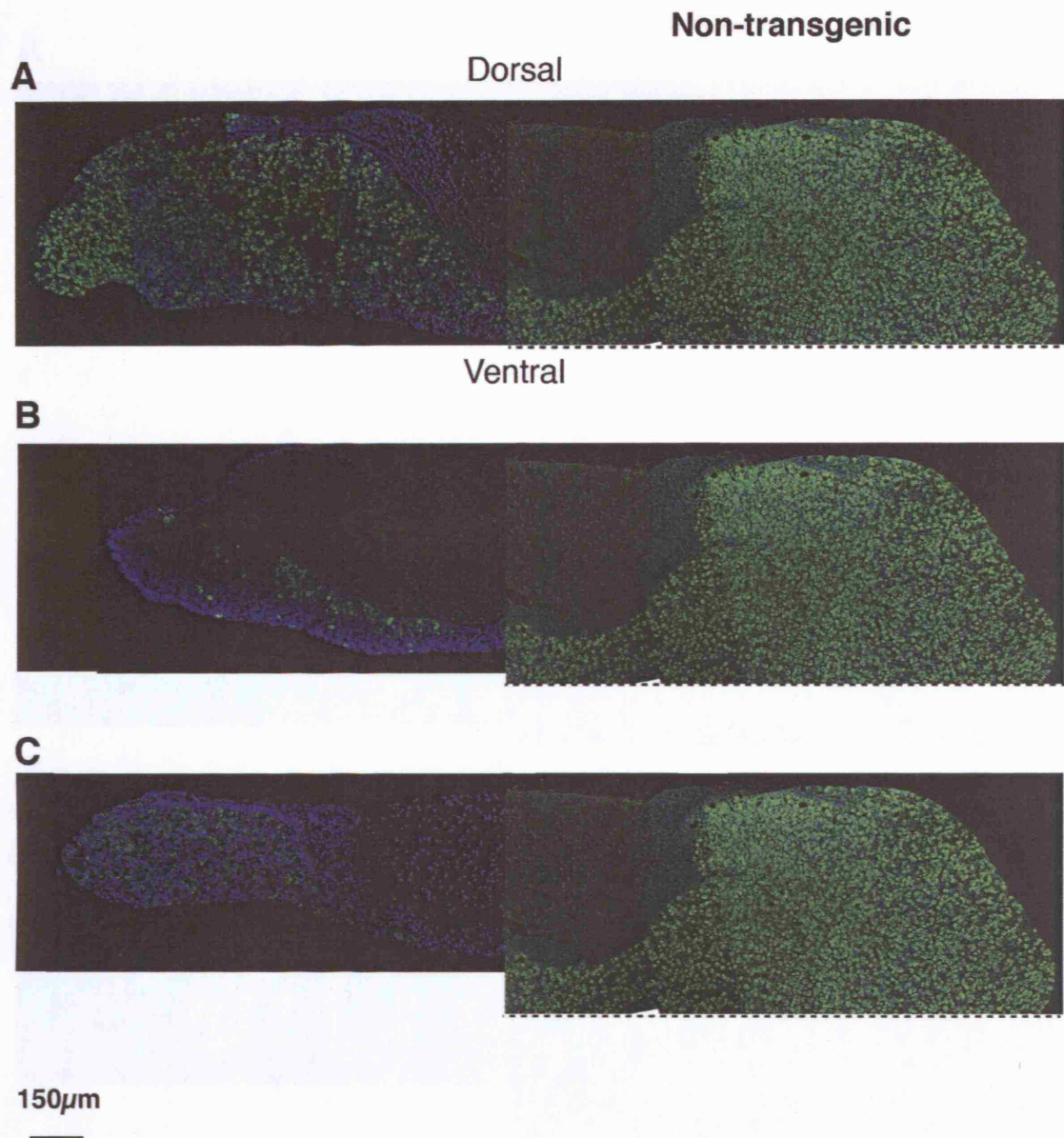


Figure 5.1 Immunofluorescence for GH in pituitary sections

Coronal sections of pituitaries from males of the low (A) and medium (B) copy lines and a female from the high copy line (C) lines were stained for GH (green), and counterstained with DAPI (blue) prior to mounting. A non-transgenic male pituitary (right-hand column) was used as a control. A mosaic of images were taken using an inverted confocal microscope and aligned in Photoshop.

The non-transgenic pituitary is bigger than shown here. The dashed line indicates the point of cut-off. The dorsal-ventral axis of the pituitary is illustrated on image A.

Scale bar = 150µm

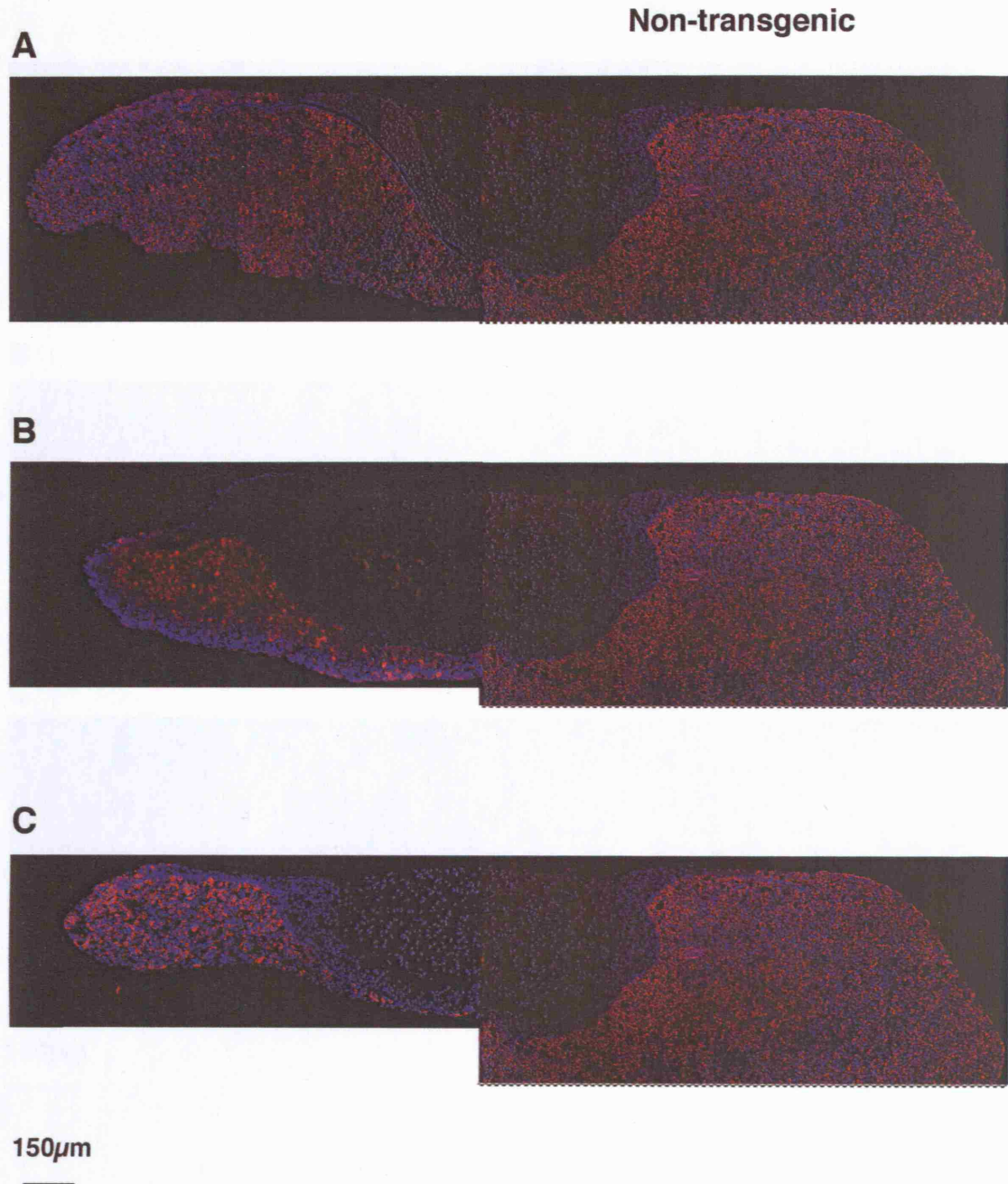


Figure 5.2 Immunofluorescence for PRL in pituitary sections

Coronal sections of pituitaries from males of the low (A) and medium (B) copy lines and a female from the high copy line (C) lines were stained for PRL (red), and counterstained with DAPI (blue) prior to mounting. A non-transgenic male pituitary (right-hand column) was used as a control. A mosaic of images were taken using an inverted confocal microscope and aligned in Photoshop. Contrast has been altered to aid viewing.

The non-transgenic pituitary is bigger than shown here. The dashed line indicates the point of cut-off.

Scale bar = 150 μ m

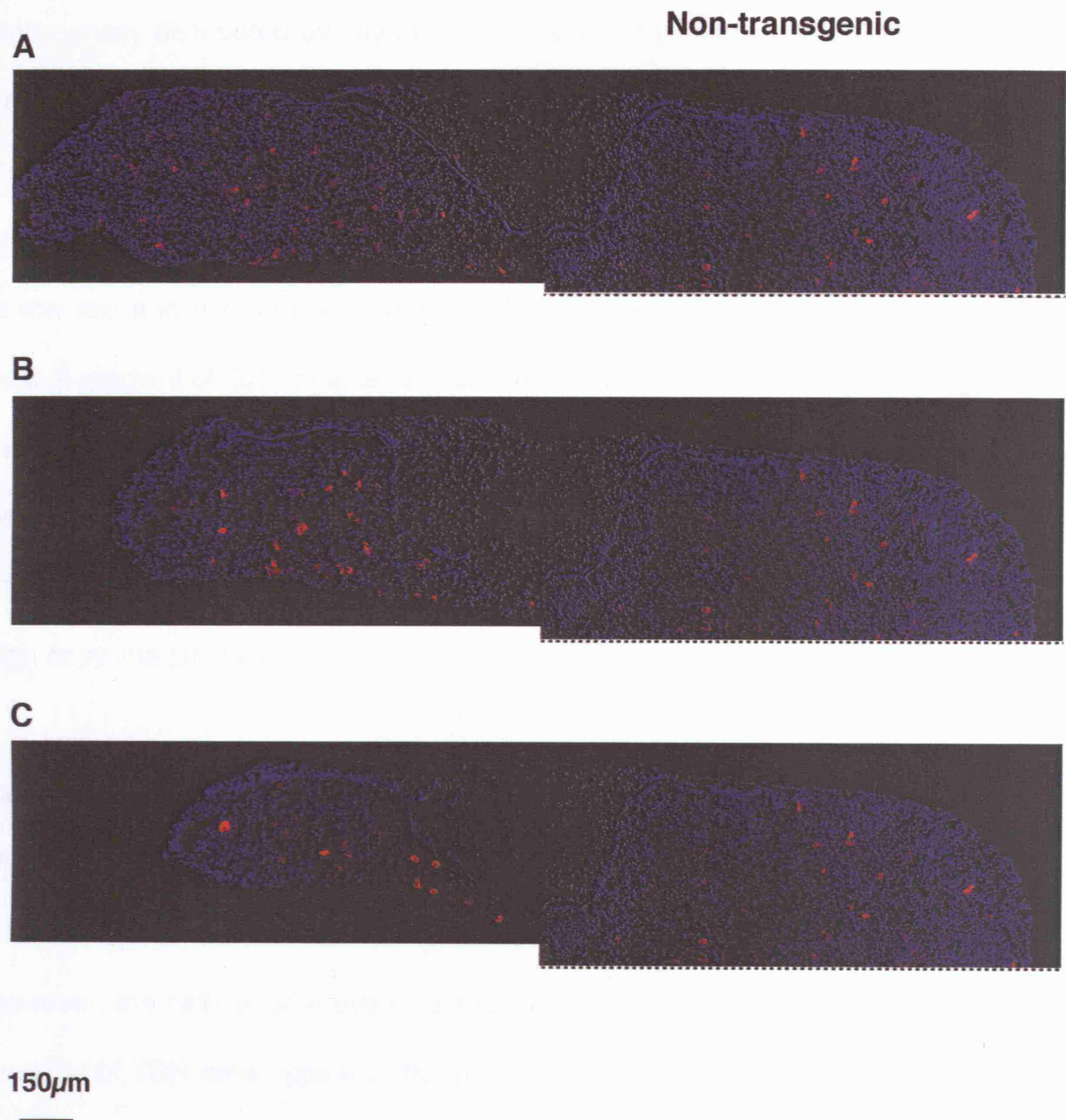


Figure 5.3 Immunofluorescence for TSH in pituitary sections

Coronal sections of pituitaries from males of the low (A) and medium (B) copy lines and a female from the high copy line (C) lines were stained for TSH (red), and counterstained with DAPI (blue) prior to mounting. A non-transgenic male pituitary (right-hand column) was used as a control. A mosaic of images were taken using an inverted confocal microscope and aligned in Photoshop. Contrast has been altered to aid viewing.

The non-transgenic pituitary is bigger than shown here. The dashed line indicates the point of cut-off.

Scale bar = 150μm

high density distributed evenly across the anterior pituitary similar to the non-transgenic male shown in the right hand panel of figure 5.1. The cell number is less than that seen in an age-matched male pituitary. The high copy line female exhibits severe anterior pituitary hypoplasia with few GH cells remaining. In the low and medium copy transgenic lines there appears to be a dorsal-ventral gradient of GH cell ablation, as there seem to be more cells remaining at the dorsal side of the anterior pituitary. In the medium copy line the remaining cells are grouped with the majority of cells lining the intermediate lobe. This dorsal-ventral pattern is not obvious from the GH immunofluorescence in the high copy line pituitary.

Consecutive sections from the same pituitaries were then stained for PRL (figure 5.2) and TSH (figure 5.3). The PRL cell density, and possibly cell number, appears to decrease in line with the degree of pituitary hypoplasia. However, the cells appear evenly distributed across the anterior pituitary. The majority of TSH cells appear to be localised to the ventral side of the anterior pituitary, with a few cells appearing between the ventral and dorsal surfaces, in both transgenic and non-transgenic mice. The cells do not group and are dispersed along this distribution gradient.

5.2.2 2-photon imaging of GH-M2 pituitaries

In order to obtain a broader view of the structure of the affected GH-M2 pituitaries 2-photon imaging was used. 2-photon imaging utilises a longer frequency, lower energy infrared excitation wavelength. Infrared photons are emitted in pulses and focused through the microscope lens to excite a

fluorophore. This longer wavelength excitation causes less damage to the sample and it is possible to image deeper into the sample than with high-energy confocal microscopy. This enables a more complete three-dimensional image of the remaining cells within the transgenic pituitary to be constructed and therefore allows study of the effects of transgene expression on the network structure.

GH-M2 mice from the low and medium copy lines were crossed to GH-GFP mice to allow imaging of the GH cell network, without the need to immunolabel the GH cells. Whole pituitaries were imaged to a depth of approximately $120\mu\text{m}$ from the ventral side, in both lateral and median zones, before sectioning and imaging of coronal sections. An age range of mice was studied to cover postnatal development, puberty and young adult. The ages used were p15, p30, p40, p50 and p60. I chose to begin by studying male mice as it is thought that they have a more distinct pattern of network development (Bonnetfont et al., 2005). Figure 5.4 illustrates the descriptions used for network formation – pituitary orientations (A and B), single cell (C), string of cells (D), cluster of cells (E) – and I will refer to these from here on. A single cell at the extreme of the z-stack cannot be considered a true single cell as it is possible that it has connections to other cells outside the image plane.

Figure 5.5 illustrates the median (zone around the pituitary stalk) and lateral zones of the ventral surface of pituitaries from a GH-GFP positive (M2 negative) male, 15 days (A-C), low copy line male, 22 days (D-F), and a medium copy line

male at 15 days (G-I). I have not yet taken a low copy line male pituitary younger than 22 days for imaging. However, this is not ideal as puberty is thought to commence at approximately 21 days in mice, around the age of weaning. From this age an increase in GH cell number and connectivity has been observed (Bonnetfont et al., 2005). It will be necessary to image the pituitary from a 15-day-old low copy line mouse at a later stage for true comparison.

At 15 days the GH-GFP pituitary appears to be tightly packed with GH cells, with no visible difference between the median (A) and lateral (B and C) zones. The cells form small strings and clusters but do not appear to form a completely connected network at this stage as some single cells can still be seen. The majority of the cells seem to be characteristically round and have a similar level of fluorescence intensity.

There are fewer GH-GFP positive cells in the low copy line pituitary than in the non-transgenic control, as seen previously with immunofluorescence. It seems as though there are more isolated single cells in the median (D) than in the lateral (E and F) zones. Some dispersed strings and small clusters can be seen in the median zone. In the lateral zone, cells are arranged in strings and small clusters, with few isolated single cells. Overall the density of GH cells appears decreased compared to the GH-GFP control mouse.

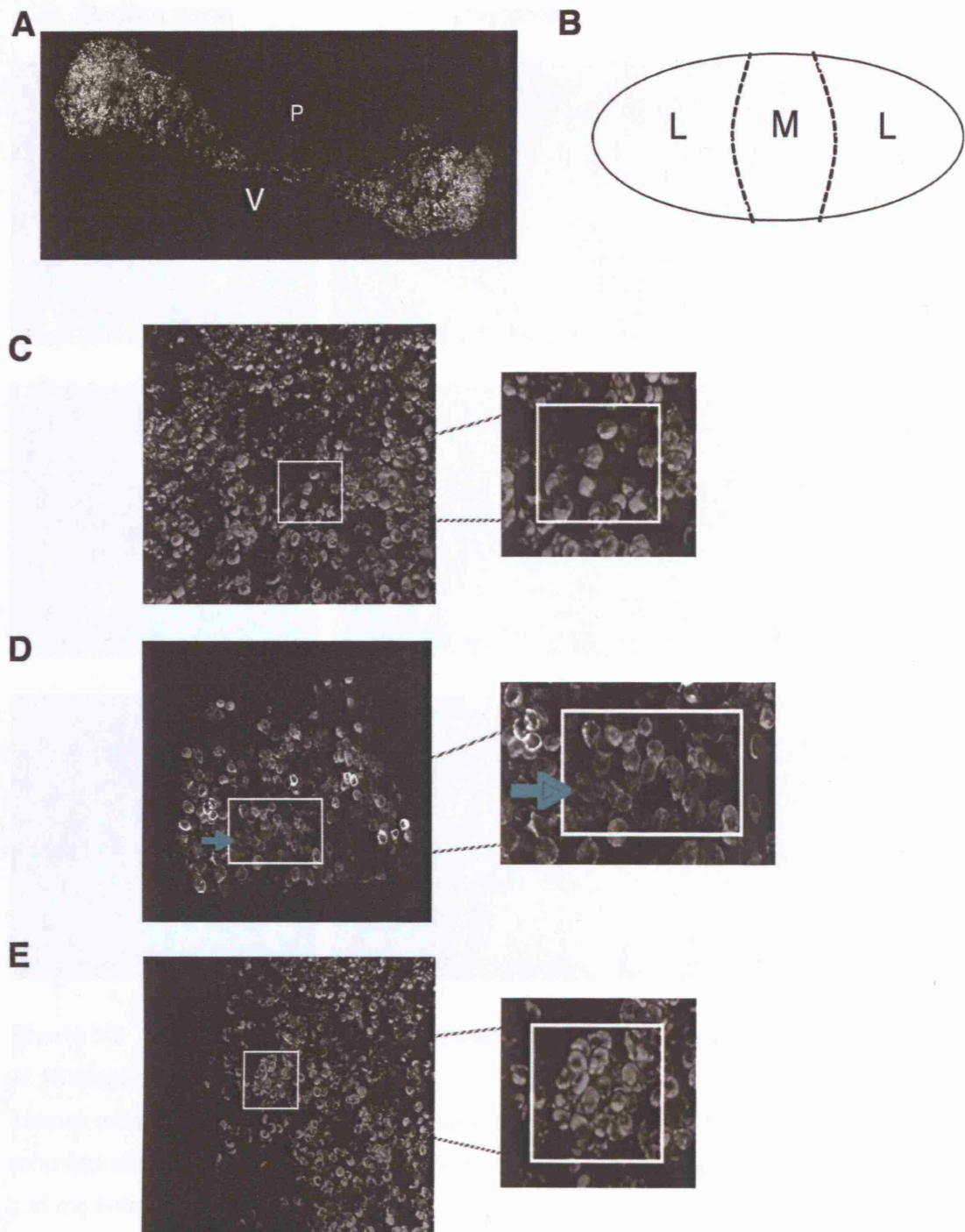


Figure 5.4 Examples of 2-photon image description

A Pituitary section; P = posterior lobe, V = ventral surface

B Zones of ventral surface; L = lateral, M = median

The white box marks the structure to describe the following:

C Single cell

D String of cells; the arrow marks the beginning of the string

E Cluster of cells

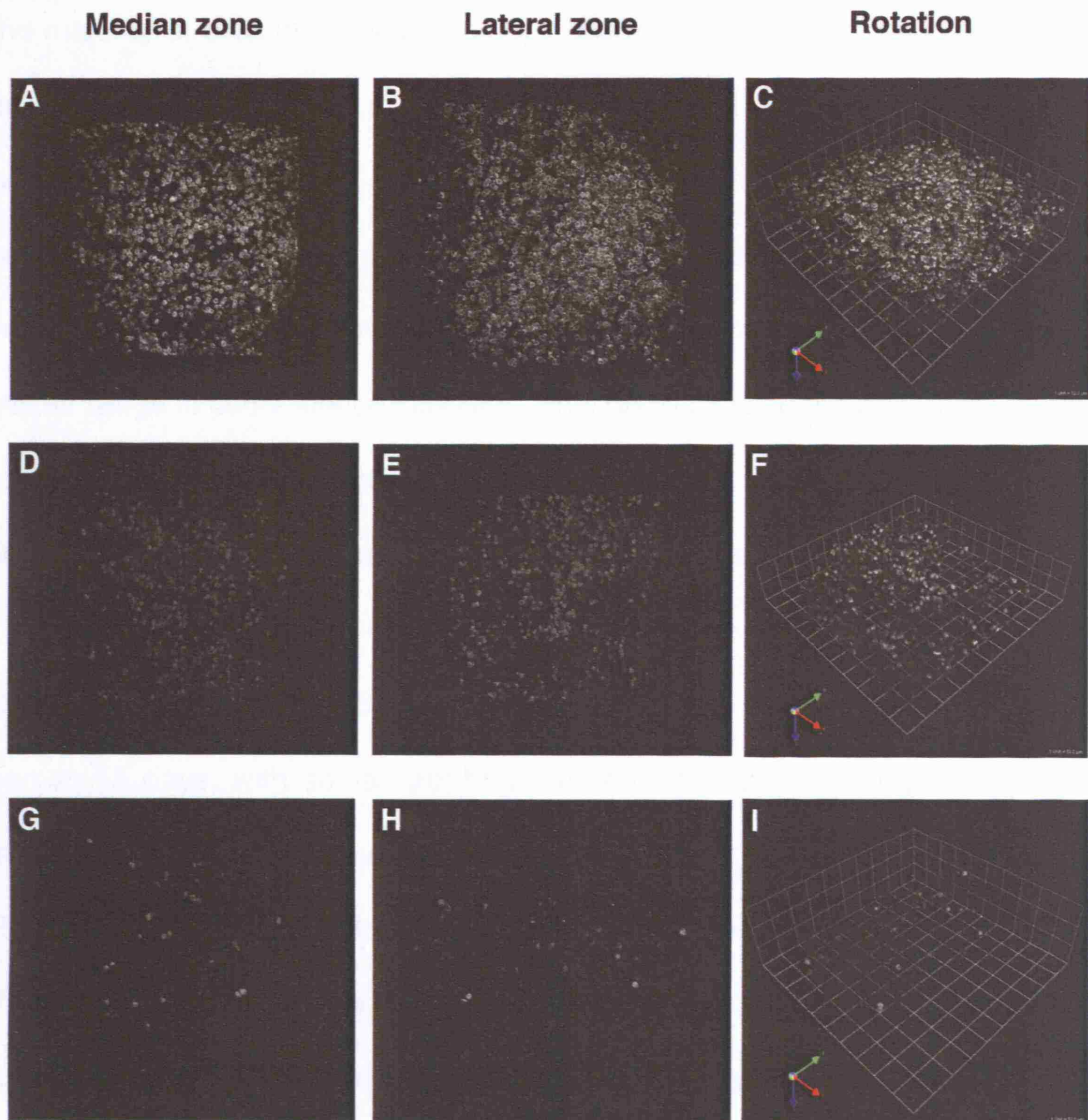


Figure 5.5 2-photon imaging of somatotrophs in GH-M2 x GH-GFP male mice at 15 days of age

Images were taken of the median and lateral zones of the ventral surface of the whole-mounted pituitary at 15 days for GH-GFP (A-C), low copy (22 days) x GH-GFP (D-F) and medium copy x GH-GFP (G-I).

- (A) NT – median zone
- (B) NT – lateral zone
- (C) NT – rotation of (B)
- (D) Low copy – median zone
- (E) Low copy – lateral zone
- (F) Low copy – rotation of (E)
- (G) Medium copy – median zone
- (H) Medium copy – lateral zone
- (I) Medium copy rotation of (H)

The majority of cells in the medium copy line male pituitary are isolated single cells, however some small groups of cells remain. It is not clear if these are remnants of strings formed at an earlier age. There are fewer cells remaining than in the low copy line pituitary. The majority of these cells have an irregular shape compared to those of the non-transgenic control and appear to exhibit a greater range in fluorescence intensity. This could indicate the poor state of the cell with a change to the storage/secretion balance of GH-GFP. Again, no difference is observed between the median (G) and lateral (H and I) zones.

At 30 days of age (figure 5.6) the control male pituitary appears more organised than at 15 days, with somatotrophs arranged into defined strings and small clusters in both the median (A) and lateral (B and C) zones, as expected (Bonnetfont et al., 2005). Some single cells can still be observed at the centre of the z section. In the low copy line pituitary (D-F) small clusters and strings can be found but these are isolated, suggesting ablation of connecting cells. There seem to be fewer cells in the median zone (D) and the cell structures in this zone are more dispersed. Figure 5.7 G-I illustrates the medium copy line male pituitary at 30 days. Small, isolated groups and strings of cells can be seen. No difference is observed between the median (G) and lateral (H and I) zones.

Figure 5.7 shows an example of the affected somatotroph population in GH-M2 x GH-GFP females, compared to the male pituitary, at 30 days. Age range imaging for somatotrophs in females remains to be completed. However, in both non-transgenic (5.7A and B) and low copy line (5.7D and E) ventral zones

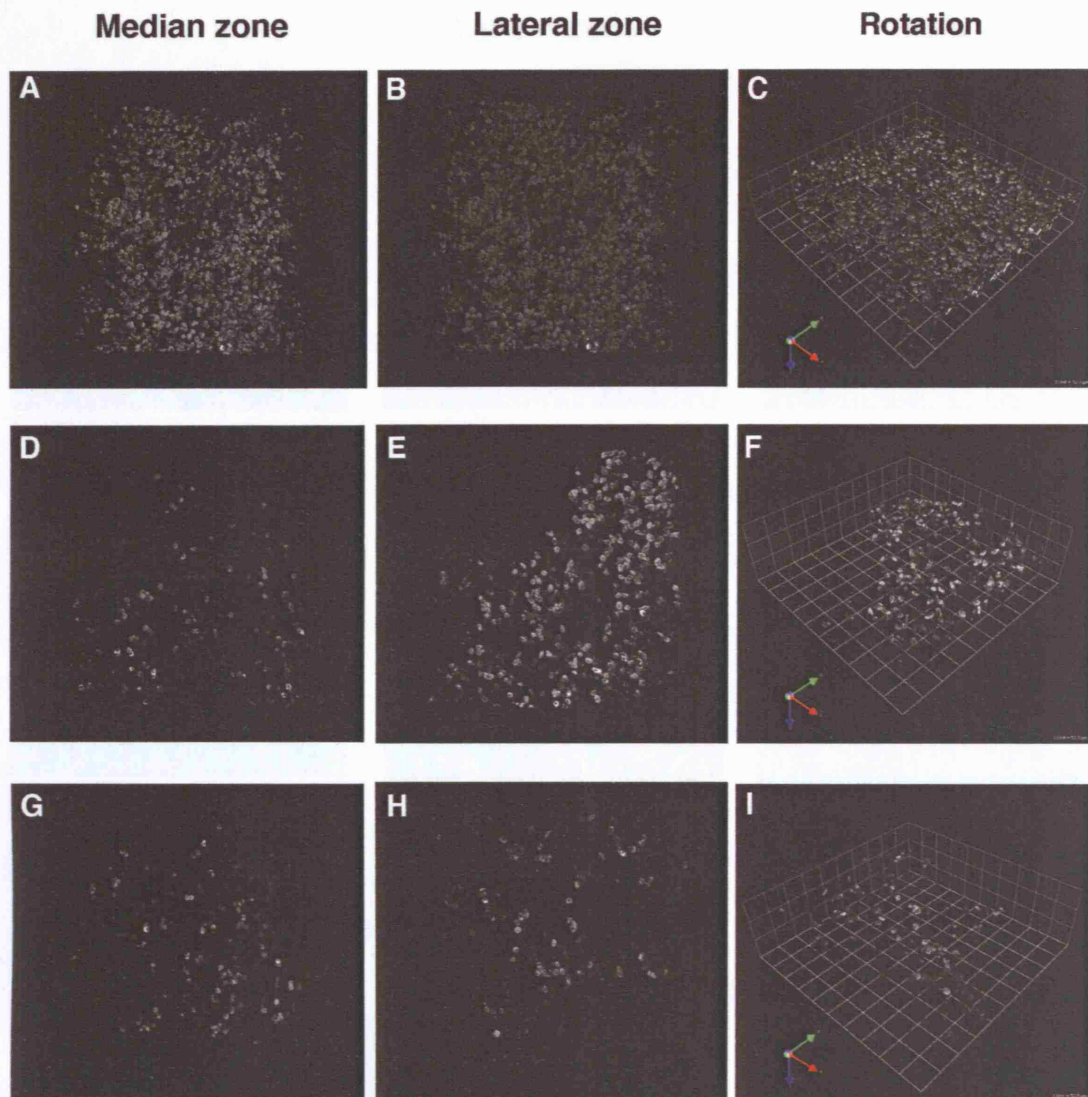


Figure 5.6 2-photon imaging of somatotrophs in GH-M2 x GH-GFP male mice at 30 days of age

Images were taken of the median and lateral zones of the ventral surface of the whole-mounted pituitary at 30 days for GH-GFP (A-C), low copy x GH-GFP (D-F) and medium copy x GH-GFP (G-I).

- (A) NT – median zone
- (B) NT – lateral zone
- (C) NT – rotation of (B)
- (D) Low copy – median zone
- (E) Low copy – lateral zone
- (F) Low copy – rotation of (E)
- (G) Medium copy – median zone
- (H) Medium copy – lateral zone
- (I) Medium copy rotation of (H)

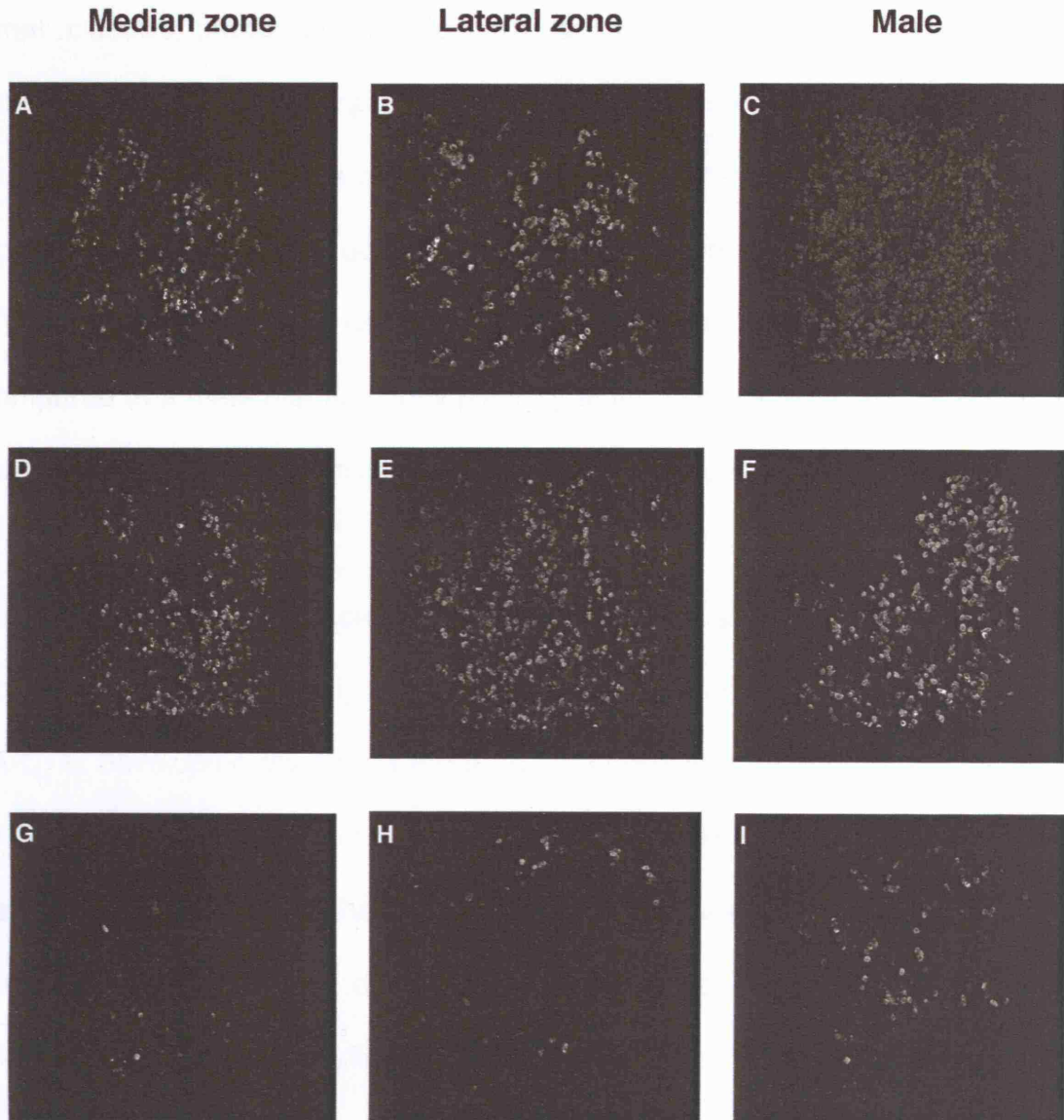


Figure 5.7 2-photon imaging of somatotrophs in GH-M2 x GH-GFP female mice at 30 days of age

Images were taken of the lateral zone of the ventral surface of the whole-mounted pituitary.

- (A) NT – median zone
- (B) NT – lateral zone
- (C) NT – male 30 days; lateral zone
- (D) Low copy – median zone
- (E) Low copy – lateral zone
- (F) Low copy – male 30 days; lateral zone
- (G) Medium copy – median zone
- (H) Medium copy – lateral zone
- (I) Medium copy – male 30 days; lateral zone

small clusters, strings and single cells can be seen. This is similar to that observed in males (5.7C and F), although the cells are less densely packed and it appears that the groups of cells (strings and clusters) are more dispersed, possibly as a result of the density. In the medium copy line pituitary (5.7G and H) there are a lot fewer cells than in the low copy and control mice, and compared to a male medium copy pituitary at the same age (5.7I). As with the male pituitary the cells form isolated, dispersed small groups and single cells.

Figure 5.8 shows the affected somatotroph network at 40 days of a GH-GFP control and a medium copy line pituitary. The structure of the control pituitary (A-C) is developing towards a more homogenous formation of large clusters, with the largest clusters forming in the lateral zone (B and C). Strings of cells can still be seen between the grouping cells. At this age an increase in volume-to-surface ratio has been observed in the lateral, but not the median, zone (Bonnetfont et al., 2005), indicating increased clustering in the lateral zone of the anterior pituitary during sexual maturation. In the medium copy line male pituitary (D-F) clusters appear to be developing in a similar way to the control pituitary, however the structures imaged are not linked by strings of cells and remain isolated. The size and number of the cell clusters appears to have increased from 30 days, although no accurate assessment has been made of this. It appears as though this effect is greater in the lateral zone (E and F). More single isolated cells can be seen in the medium copy line than in the control pituitary. The extended shape of the cells in the z projection (F) indicates either incorrect deconvolution of the initial image or movement of the sample during acquisition.

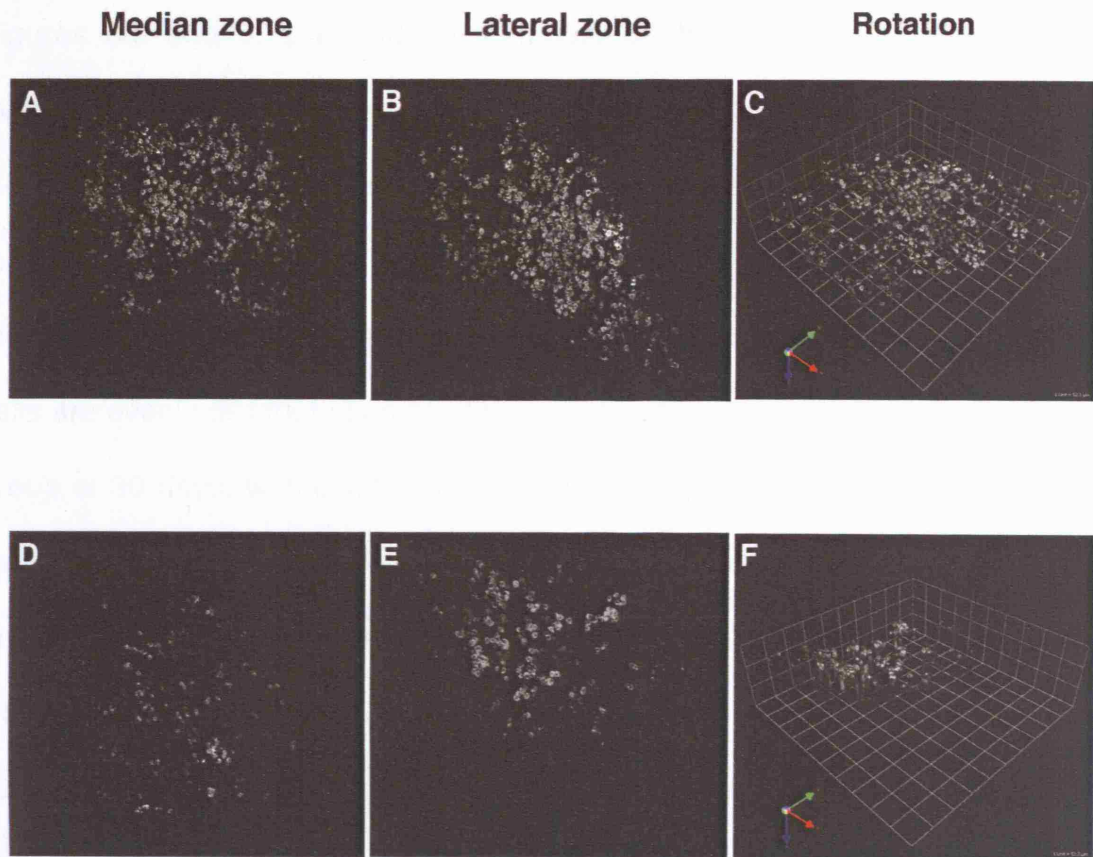


Figure 5.8 2-photon imaging of somatotrophs in GH-M2 x GH-GFP male mice at 40 days of age

Images were taken of the median and lateral zones of the ventral surface of the whole-mounted pituitary at 40 days for GH-GFP (A-C) and medium copy x GH-GFP (D-F).

- (A) NT – median zone
- (B) NT – lateral zone
- (C) NT – rotation of (B)
- (D) Medium copy – median zone
- (E) Medium copy – lateral zone
- (F) Medium copy – rotation of (E)

Figures 5.9 and 5.10 provide a summary of the progression of structure formation with age in low and medium copy line GH-M2 male mice compared to GH-GFP control males in the median and lateral zones, respectively. From 15 to 40 days it is possible to see the gradual formation of clusters in both the median and lateral zones in GH-GFP control animals. At 15 days strings of cells are evenly distributed across the anterior pituitary. These strings begin to group at 30 days, with clusters forming by 40 days. In low copy line GH-M2 male mice there is a more obvious median-lateral difference in GH cell network. In the median zone at 15 days there are fewer cells than the GH-GFP control. Cells are dispersed in small isolated strings and as single cells. In the medium copy line GH-M2 male pituitary there are fewer cells in both the median and lateral zones compared to low copy GH-M2 and control GH-GFP mice. The number of cells appears to increase by 30 days but the small string and single cell structures remain isolated and dispersed. Cluster formation begins at 40 days in both zones. It appears as though the clusters that form have a greater fluorescence intensity than the single cells.

Figure 5.11 shows 2-photon images of coronal sections from a GH-GFP control male pituitary (A; zoom 0.7) compared to a low copy line (x GH-GFP) male pituitary (B; zoom 0.9) at 50 days. The lateral zone of the low copy line pituitary appears to contain isolated strings and small clusters. Some dispersed single cells can also be seen in this zone. In comparison, the lateral zone of the control pituitary is densely packed with GH cells in relatively large cell clusters, as previously reported for this age (Bonnefont et al., 2005). Imaging of coronal

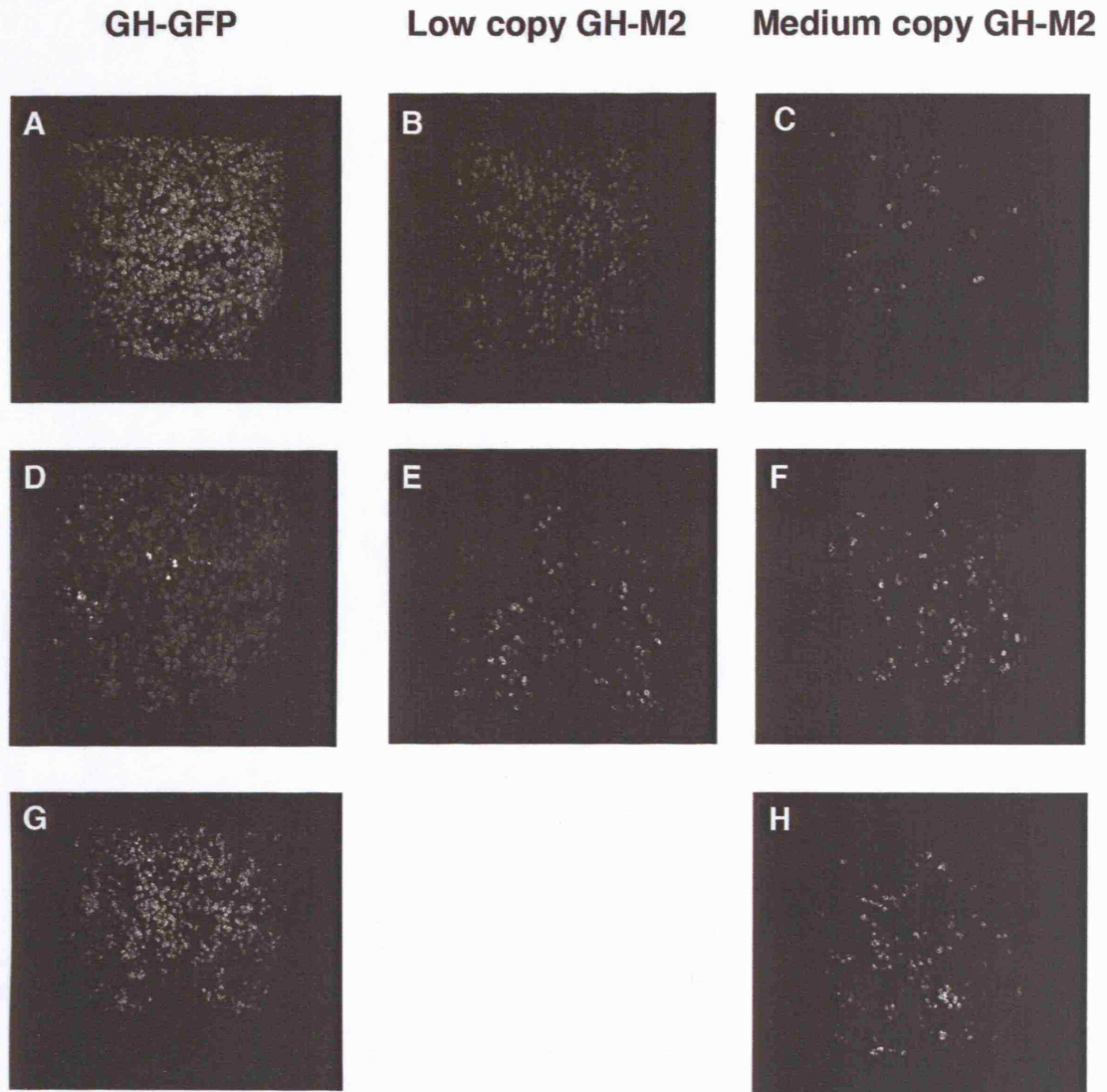


Figure 5.9 Age progression of somatotroph network ablation in the median zone of the pituitary

2-photon images of the median zone of GH-GFP, low copy and medium copy line GH-M2 mice have been collated to show network development with age at:

- (A-C) 15 days
- (D-F) 30 days
- (G-I) 40 days (GH-GFP and medium copy line only)

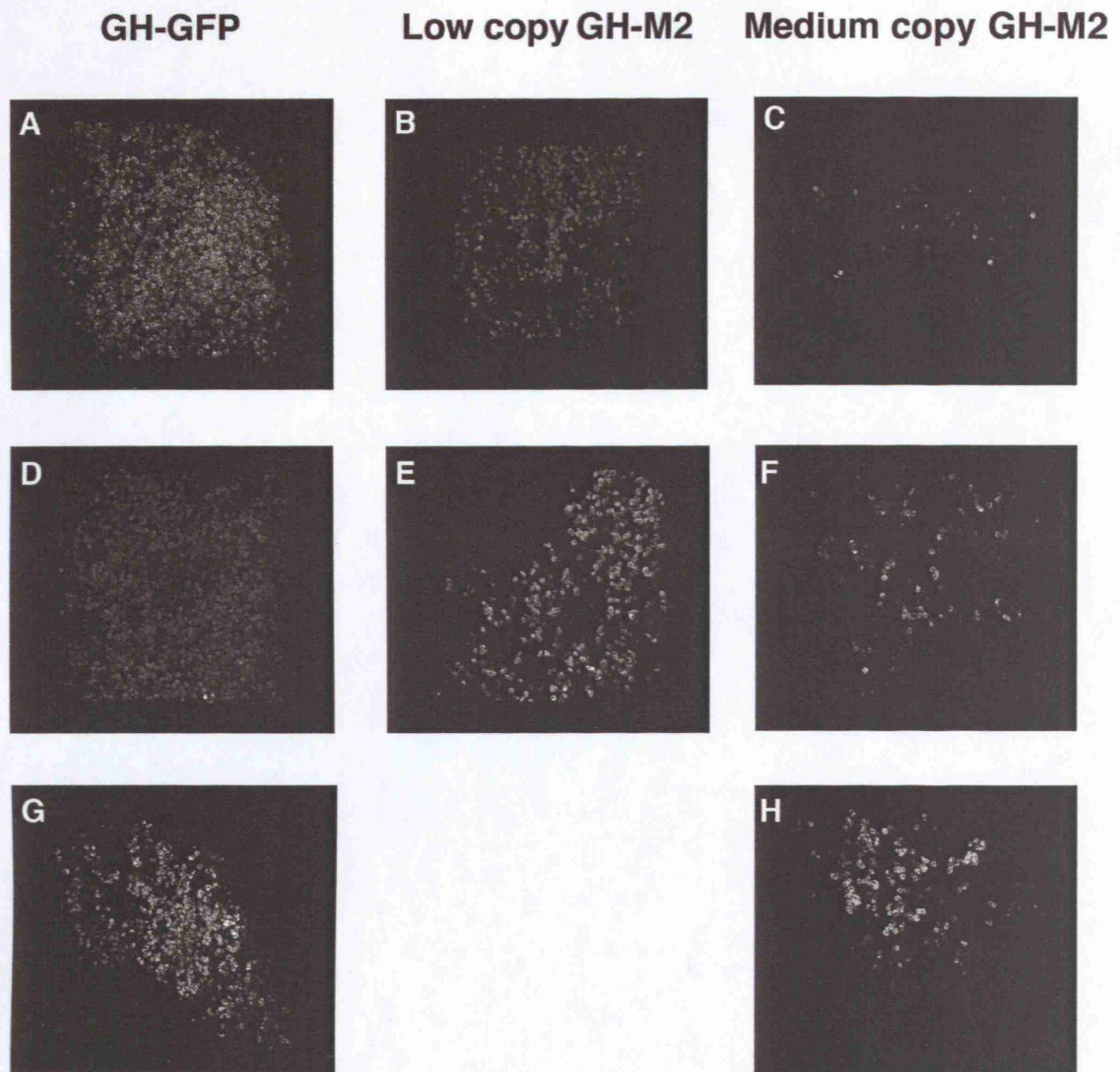


Figure 5.10 Age progression of somatotroph network ablation in the lateral zone of the pituitary

2-photon images of the lateral zone of GH-GFP, low copy and medium copy line mice have been collated to show network development with age at:

- (A-C) 15 days
- (D-F) 30 days
- (G-I) 40 days (GH-GFP and medium copy line only)

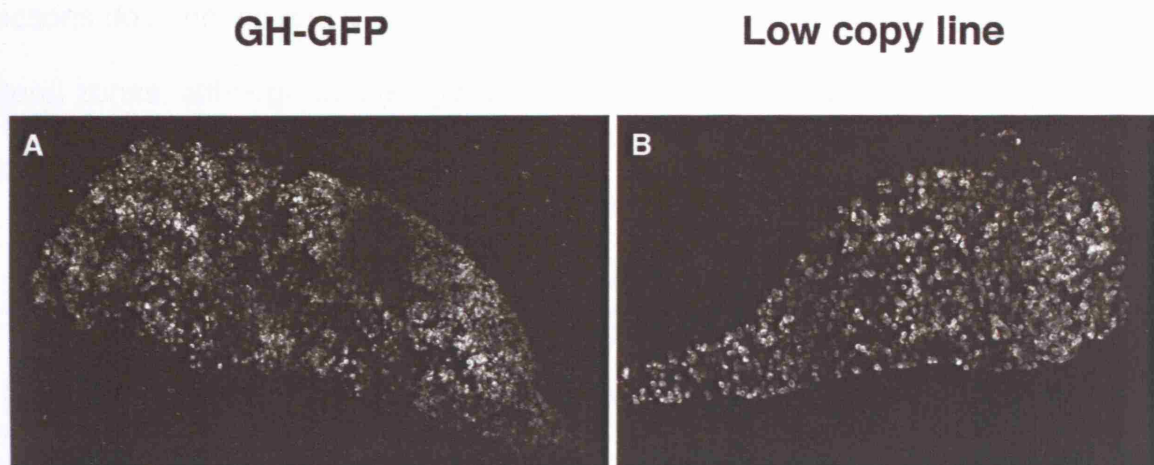


Figure 5.11 2-photon imaging of somatotrophs in GH-M2 x GH-GFP male mice at 50 days of age

A mosaic of images was taken across a 120 μ m coronal section of a GH-GFP control male pituitary (A) and a low copy line male (crossed to GH-GFP) pituitary (B) at 50 days.

sections does not enable a clear comparison to be made between median and lateral zones, although at this age a median-lateral difference in distribution is expected.

5.2.2.1 Estimation of cluster distribution and cell number

I have attempted to quantify the distribution and number of clusters in the median and lateral zones of the GH-GFP and medium copy line male pituitaries at 15 (figure 5.12) and 30 (figure 5.13) days. In order to do this a number of parameters had to be arbitrarily set. Firstly, the volume of a single cell was set to $500\mu\text{m}^3$ based on the volume of a sphere ($\frac{4}{3}\pi r^3$), estimating the diameter of a GH cell as $10\mu\text{m}$. This was the smallest diameter of single GH-GFP cells measurement from previously obtained confocal images (not shown). It was then necessary to limit the area of the image analysed to attempt to normalise the differences in area of pituitary imaged for different samples. The area was set to 200 units by 200 units (x by y). The z parameter could not be altered. This therefore introduces a degree of variation to the numbers obtained as the volume of the area analysed, and possibly cells contained within it, will vary between samples. Analysis was performed in Volocity 4.1 (Improvision) using an identical protocol for each image. The distributions described here are relative as the total area of the GH-M2 positive pituitary is much smaller than the control GH-GFP pituitary, despite an equivalent image area being selected for analysis.

Figure 5.12 Distribution and numbers of cells in clusters in median and lateral zones of non-transgenic and medium copy anterior pituitaries at 15 days

Image analysis was performed in Volocity on the images shown in figure 5.5 to generate the number of objects (clusters) of a specific size (A and B) in a defined area. The total number of cells at each cluster size was determined by dividing the cluster volume by the volume of a single cell ($500\mu\text{m}^3$) (C and D). The proportion of cells (%) found in a cluster of size x in the sample area is shown in (E and F).

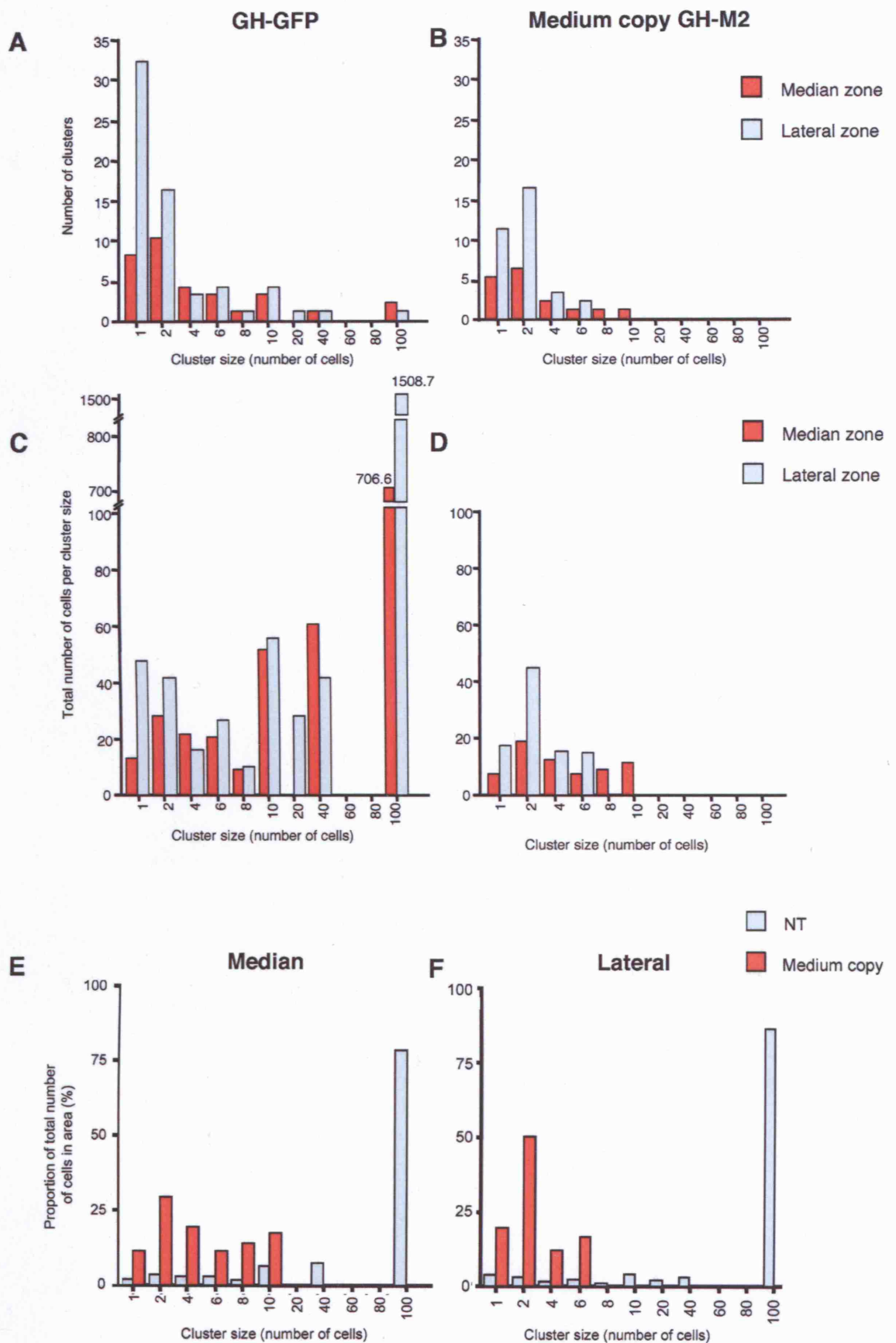


Figure 5.13 Distribution and numbers of cells in clusters in median and lateral zones of non-transgenic and medium copy anterior pituitaries at 30 days

Image analysis was performed in Volocity on images shown in figure 5.6 to generate the number of objects (clusters) of a specific size (A and B) in a defined area. The total number of cells at each cluster size was determined by dividing the cluster volume by the volume of a single cell ($500\mu\text{m}^3$) (C and D). The proportion of cells (%) found in a cluster of size x in the sample area is shown in (E and F).

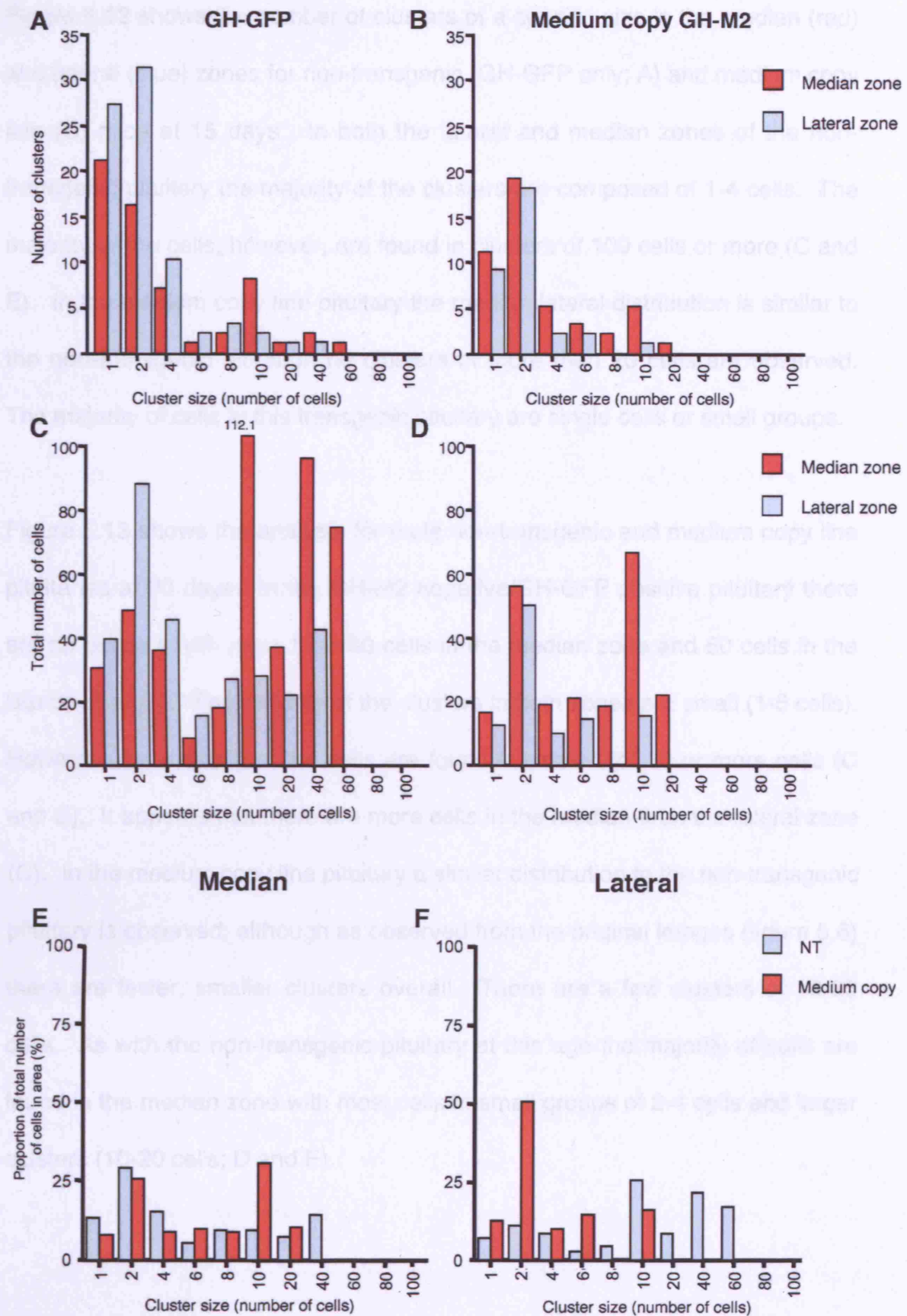


Figure 5.12 shows the number of clusters of a specific size in the median (red) and lateral (blue) zones for non-transgenic (GH-GFP only; A) and medium copy line (B) mice at 15 days. In both the lateral and median zones of the non-transgenic pituitary the majority of the clusters are composed of 1-4 cells. The majority of the cells, however, are found in clusters of 100 cells or more (C and E). In the medium copy line pituitary the median-lateral distribution is similar to the non-transgenic although no clusters of more than 20 cells are observed. The majority of cells in this transgenic pituitary are single cells or small groups.

Figure 5.13 shows the analysis for male non-transgenic and medium copy line pituitaries at 30 days. In the GH-M2 negative/GH-GFP positive pituitary there are no clusters with more than 80 cells in the median zone and 60 cells in the lateral zone (A). The majority of the clusters in both zones are small (1-6 cells). However, the majority of the cells are found in clusters of 10 or more cells (C and E). It appears that there are more cells in the median than the lateral zone (C). In the medium copy line pituitary a similar distribution to the non-transgenic pituitary is observed, although as observed from the original images (figure 5.6) there are fewer, smaller clusters overall. There are a few clusters of 10-20 cells. As with the non-transgenic pituitary at this age the majority of cells are found in the median zone with most cells in small groups of 2-4 cells and larger clusters (10-20 cells; D and E).

5.2.2.2 Relationship of remaining somatotrophs to pituitary capillary system

Figure 5.14 shows blood vessel staining in a 55-day-old low copy line male pituitary. Gelatin containing the fluorescent dye rhodamine was perfused through the mouse prior to fixation to indicate the blood vessels of the pituitary, enabling imaging of the remaining GH cells in relationship to the capillary system. From the ventral images (A and B) it is possible to see that the majority of the remaining cells are localised in close proximity to the blood vessels. There are some cells that appear isolated (arrow - A) from the blood vessels, but upon rotation of the original image (arrow - B) it is possible to see that it is part of a larger cell cluster close to a capillary. Figure 5.14 C shows an area of higher magnification. Upon rotation of this image (D) it is possible to detect individual cells that are isolated from the capillary network, as marked by the arrow. The 120 μ m coronal section (E) shows that the blood vessels are enveloped by GH cells. This imaging implies that the majority of the remaining cells are theoretically able to secrete GH into the capillary blood supply upon stimulation.

5.2.2.3 GH cell network in a model of secondary GH deficiency

In the GHRH-M2 mouse the observed GH deficiency is secondary to the ablation of GHRH neurons (Le Tissier et al., 2005). To provide a comparison of cell network development to the GH-M2 model, where M2 channel activity directly affects GH cell function and network structure, GHRH-M2 mice are also being imaged. It is hoped that this will provide clues to the structures observed

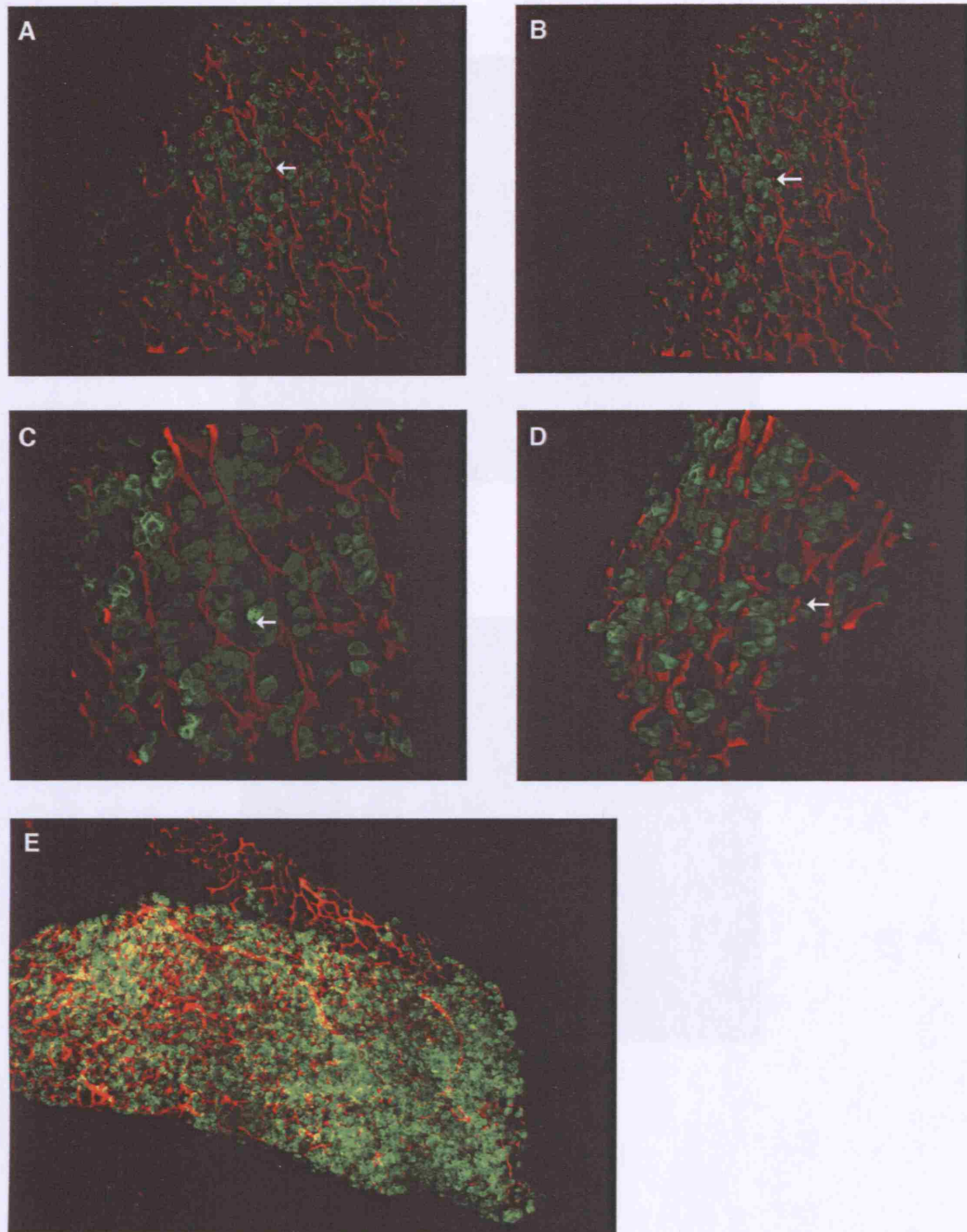


Figure 5.14 2-photon imaging of a low copy line male pituitary perfused with gelatin/rhodamine

A 55-day-old low copy male mouse (crossed to G H-GFP) was perfused with a gelatin/rhodamine solution to label the blood vessels.

- (A) ventral view
- (B) rotation of view (A)
- (C) ventral view – 2x zoom
- (D) rotation of view (C)
- (E) 120 μ m coronal section

Arrows indicate cells that appear isolated.

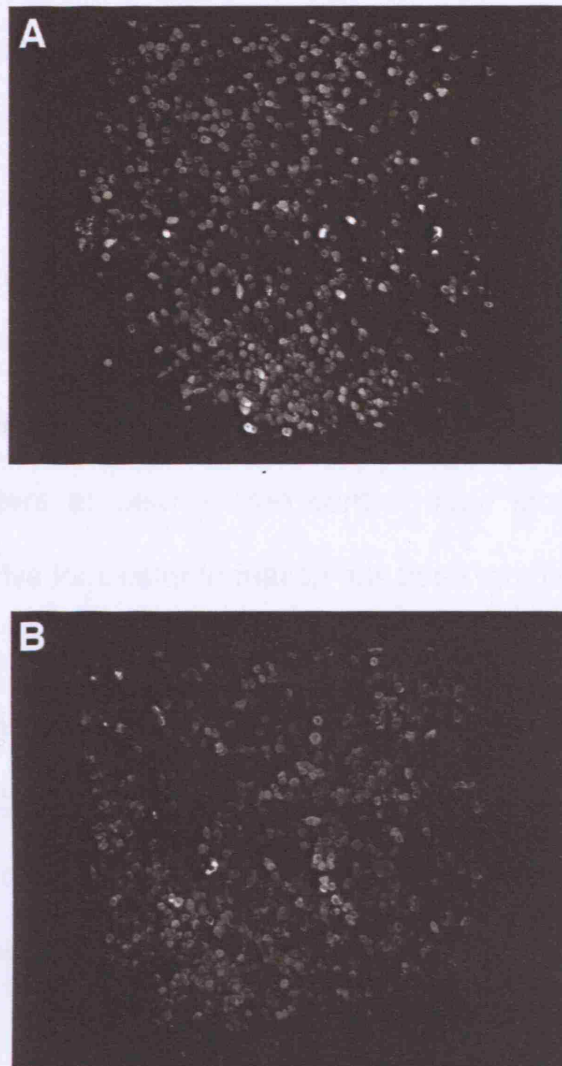


Figure 5.15 An example of the somatotroph network in male and female GHRH-M2 mice at 55 days

2-photon microscopy imaging of the lateral zone of the ventral surface of whole-mounted pituitaries.

- (A) GHRH-M2 x GH-GFP male
- (B) GHRH-M2 x GH-GFP female

in the GH-M2 pituitary. GHRH-M2 mice have also been crossed to GH-GFP to aid imaging of the pituitary and to allow direct comparison with the foregoing analysis of GH-M2 mice.

Figure 5.15 shows an example of a male and female transgenic pituitary at 55 days. It is possible to see that in the absence of GHRH the male and female pituitaries have a similar network structure of single cells and strings of cells. There are no clusters as seen in the control male mouse (figure 5.11B) indicating that the drive for cluster formation has been removed.

5.2.2.4 Imaging the secondary effects of somatotroph ablation

Immunohistochemistry for PRL in low and medium copy line pituitaries indicated an effect on the lactotroph population when compared to a non-transgenic control. GH-M2 mice have therefore been crossed to the Prl-DsRed line to enable imaging of the effects of somatotroph ablation on the lactotroph population.

An example of this is shown in figure 5.16. Here, a pituitary from a medium copy line male at 70 days and a Prl-DsRed positive (M2 negative) littermate have been imaged in the lateral zone of the ventral surface. When imaging Prl-DsRed it is necessary to increase the excitation level of the laser to ensure that all areas of these irregular shaped cells can be detected. However, this means that a high level of background is captured with the image making analysis more difficult. Also, it is known from immunohistochemical studies of dispersed

Prl-DsRed anterior pituitaries that in male mice not all of the lactotroph population is detectable with the Prl-DsRed construct. Taking these caveats into account, it still appears that the medium copy line pituitary contains less, more dispersed PRL cells. It is planned to perform an age range imaging study for male and female low and medium copy GH-M2 x Prl-DsRed mice to provide a clearer idea of the effects of GH-M2 expression on the PRL cell population. Ideally, this will be done alongside the GH imaging by using low and medium copy line mice crossed to both GH-GFP and Prl-DsRed.

5.2.2.5 Imaging of high copy line pituitaries

It has not been possible to image high copy line pituitaries in the same way, from p15 to p60, because of their poor breeding ability. An example of a high copy line female pituitary at 55 days can be seen in figure 5.17 (B and D). This female is transgenic for GH-M2, GH-GFP and Prl-DsRed. No control pituitary was available at the time of imaging but previous studies by the Mollard lab indicate a close relationship between the somatotroph and lactotroph populations (A and C). There are very few GH cells remaining in the high copy pituitary; for example only five GH-GFP positive cells can be seen in 5.17D. There are a greater number of Prl-DsRed cells present and some that could be positive for both GH and PRL, suggesting that a PRL deficiency could be secondary to the ablation of somatotrophs (see Chapter 7 for further discussion). It remains to be seen, however, if the number of double positive cells is greater in the high copy line than either the low or medium copy line

A



B



Figure 5.16 Medium copy male GH-M2 x PrI-DsRed at 70 days

2-photon imaging of the lateral zone of the ventral surface of:

- (A) PrI-DsRed male
- (B) GH-M2 x PrI-DsRed male

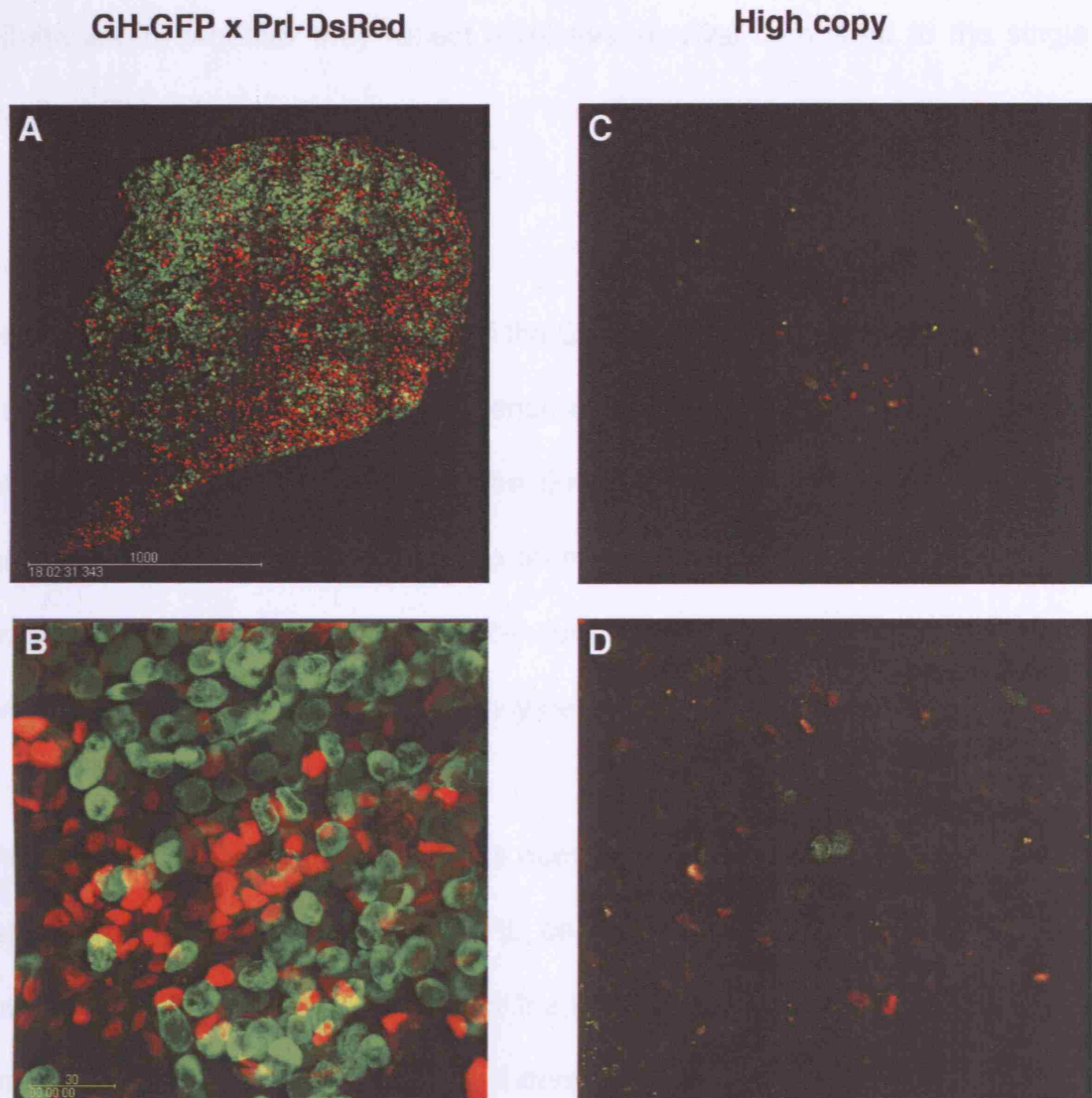


Figure 5.17 Ventral images from a high copy line female x GH-GFP and Prl-DsRed at 55 days

2-photon images of two lateral zones (A and B) of a high copy line female pituitary.

- (A) GH-GFP x Prl-DsRed female (image kindly contributed by Lafont, Mollard, Le Tissier)
- (B) zoom of GH-GFP x Prl-DsRed female (image kindly contributed by Lafont, Mollard, Le Tissier)
- (C) 25x objective; 1.5x zoom
- (D) 25x objective; 1x zoom

pituitaries, or whether they reflect a relative survival compared to the single positive GH cells.

5.3 DISCUSSION

Hypoplasia of the anterior pituitary of the GH-M2 mice increases in severity with copy number. The immunofluorescence experiments shown here indicate the effects of GH-M2 expression on the GH, PRL and TSH cells of the adult pituitary of each line. From these experiments it is clear that the somatotroph population is severely affected by M2-induced silencing of function and ablation, which correlates with the GH deficiency detected by RIA (chapter 3).

Although formal quantification of cell number has not been done, the number and density of immunopositive PRL cells appears to decrease with copy number, and may in turn account for the PRL deficiency measured in medium and high copy line mice. Previous models of somatotroph ablation have also reported PRL deficiencies. For example, expression of thymidine kinase (Borrelli et al., 1989) and diphtheria toxin (Behringer et al., 1988) in somatotrophs observed an almost complete depletion of the PRL cell population as assessed by immunofluorescence. These groups did not report any adverse effects on other pituitary cell types. RIA for TSH (chapter 3) indicated that medium and high copy line mice have significantly reduced levels of pituitary TSH. Immunofluorescence for TSH suggests that there is a decrease in thyrotroph cell number with copy number, although accurate cell counts are needed. A severe deficiency of GH, PRL and TSH cells was

reported in the Snell dwarf mouse (Roux et al., 1982; Yashiro et al., 1988) that is known to be caused by a mutation in Pit-1 (Li et al., 1990). It is possible that ablation of somatotrophs has a knock-on effect on lactotroph and thyrotroph number. For example, GH deficiency is likely to result in an increase in GHRH production (Mizobuchi et al., 1991; Phelps et al., 1993). This in turn will drive the proliferation of the existing somatotroph population and can stimulate differentiation of precursor cells to GH cells (Billestrup et al., 1986; Burton et al., 1991; Soto et al., 1995). Continuous stimulation of this pathway may lead to a depletion of precursor cells that differentiate to PRL and TSH cells in the developing and adult pituitary. It may be possible that a decrease in the number of PRL and TSH cells is a result of cell death induced by the release of cytokines from ablated somatotrophs and invading macrophages. This effect was observed in the autosomal dominant GH deficiency mouse model (exon3 deletion; (McGuinness et al., 2003)), who suggested a heightened macrophage response to somatotroph cell death was responsible for the other hormone deficiencies recorded. Further pituitaries will need to be assayed, and counts performed, to assess these observations. The total number and density of PRL and TSH cells appear to decrease with copy number and the subsequent anterior pituitary hypoplasia. This could be the reason for the reduced pituitary contents of these hormones in the medium and high copy lines, as discussed in chapter 4. It will be interesting to see if a similar effect is seen in LH or ACTH cells, as these are generated from an unrelated cell lineage (Scully and Rosenfeld, 2002).

The high copy line pituitary used is from a female, whereas the other lines are male. This is due to the very low number of animals produced from this line. Ideally a direct comparison would have been made between one gender. The severe hypoplasia of the high copy line anterior pituitary is clearly evident.

2-photon imaging was used to analyse the effect of GH-M2 transgene expression on the GH cell network throughout the pituitary. This imaging technique provides a more accurate assessment of cell ablation as it is possible to image tissue to a depth of $120\mu\text{m}$, enabling imaging of cell-cell interactions across a thick section of the anterior pituitary. This method also makes use of endogenously fluorescently tagged cells, for example by using the GH-GFP transgenic mouse, rather than relying on immunohistochemistry. A range of ages was studied in low and medium copy line mice compared to GH-GFP age-matched controls to provide an insight to how the network develops postnatally.

Imaging of the ventral surface of the anterior pituitary revealed that there is a positional difference in ablation of the GH cell network with age. In both the low and medium copy lines it appears as though the lateral zone contains more, or larger, clusters with age than the median zone. A similar characteristic was noted by Bonnefont *et al.* (2005) who concluded that the median-lateral difference was a result of greater plasticity in the lateral zones upon sexual maturation. In other words, there is a greater degree of somatotroph proliferation predicted in the lateral zones of the anterior pituitary during puberty. The positional difference of cluster formation has been reported to

disappear by 100 days of age in the male pituitary. It would be interesting to image the low and medium copy line mice at this age. If M2 ablation occurs following accumulation of the channel protein at the plasma membrane it is possible that the cells imaged in this development snapshot are newly generated cells that do not yet have sufficient levels of M2 protein to cause cell death. An *in vivo* BrdU incorporation study is planned to study the proliferative ability of the somatotroph population of low and medium copy GH-M2 mice compared to non-transgenic littermates. Alternatively, although unlikely to have occurred in all three lines, it could be that a subpopulation of cells are surviving, for example as a result of mosaic transgene expression or transgene silencing, and are functionally unaffected following the proliferative drive of GHRH. Behringer *et al.*, 1988, observed a similar pattern in their induced dwarf model. Very few GH cells remained following ablation. However, the few that were imaged were in small clusters. They suggested that this was a result of proliferation of a single cell that had escaped activation of the transgene, possibly by gene deletion, mutation or an epigenetic event.

From imaging of the medium copy male pituitary it is possible to say that it is not necessary for a complete network to be in place for cluster formation to occur. For example, at 40 days of age the medium copy line male had small, dispersed, isolated clusters despite the absence of other network structures. It is unclear, however, how this clustering occurs. There are two main possibilities: (1) the group of cells is generated from a single progenitor cell and the process of cell ablation ensures the clusters do not grow to a large size; or,

(2) single cells migrate towards others in close proximity to maximise GH secretion in response to GHRH, as it has been shown that cells in close contact with one another can mount a coordinated response to GHRH (Bonnetfont et al., 2005).

GHRH-M2 mice are a good example of a mouse model of secondary GH deficiency. Complete ablation of the GHRH neurons by M2 expression results in transgenic mice with severe GH deficiency, pituitary hypoplasia and dwarfism (Le Tissier et al., 2005). Imaging of the GHRH-M2 mice has shown that in the absence of GHRH cluster formation does not occur. The male GH cell network begins to resemble that seen in the female pituitary, removing the apparent sexual dimorphism of network formation. When these mice are given a single injection of GHRH there is a small but significant increase in circulating plasma GH (Le Tissier et al., 2005) indicating the remaining GH cells are still responsive to a GHRH stimulus. However, this is not the robust response seen in non-transgenic animals, or to the level seen in GH-M2 mice (chapter 3), suggesting clustering of cells aids the simultaneous GH secretion that results in release of a higher GH pulse into the circulation.

Despite disruption of the GH network in low copy line animals, the majority of the remaining cells are located in close proximity to the pituitary capillary network. This may help to explain the ability of the low and medium copy line animals to reach a similar body weight as non-transgenic animals by adulthood. This location would enable cells to successfully secrete GH into the circulation

in response to GHRH, in turn increasing circulating IGF-1 levels. However, before this assumption can be made it will be necessary to image female low copy as well as male and female medium copy line (x GH-GFP) pituitaries in the presence of gelatin/rhodamine, to ensure this is not unique to one group of animals.

Although I have attempted to estimate the cell number and cluster size at 15 and 30 days, accurate counts of the remaining endocrine cells of the anterior pituitary in the three lines have not been obtained. The crude analysis used here is based on the intensity and volume of objects in the selected area of the image. There is variation between the original images obtained, in the z plane, as sample and mounting quality limited the depth to which tissue could be imaged. This may skew the distribution and numbers obtained. Other possibilities such as counts of dispersed cells and tissue sections following immunofluorescence rely on the accuracy of the individual performing the experiment and counts. It would be preferable to use an automated method such as fluorescence activated cell sorting (FACS) analysis. This would enable sorting of dispersed cells based on fluorescence. However, fluorescently tagged mouse models are only available for GH and PRL. Analysis of other cell populations would require immunofluorescence on dispersed cells, which may result in some cell loss. An alternative to this would be to use a computer programme that could count the cells based on fluorescence across a pituitary section counterstained with DAPI. The Mollard group are in the process of

designing a programme for this purpose. For both methods it will be necessary to average across a number of pituitary samples from an individual line.

It has not been possible to image more than the one example of the GH cell network in high copy line mice as these mice do not breed well. However, in the example shown here there is a high level of fluorescence background. This may be attributable to a large amount of cell debris remaining following cell ablation. There also appears to be a high relative incidence of GH and PRL colocalisation in the few remaining cells, although further imaging is needed to confirm this. If this is the case it may be possible that these cells represent somatotroph and lactotroph progenitor cells or that the GH deficiency is driving transdifferentiation of lactotrophs to somatotrophs.

Further work is required to complete this imaging study and will be completed in collaboration with IGF, Montpellier. The initial development study with GH-GFP will be completed in males and females of the low and medium copy lines. This will be extended to include embryonic stages of development from e15.5, the age at which GH first appears (Japon et al., 1994). The next step will be to compare the lactotroph network of the low and medium copy line female mice to non-transgenic controls using the Prl-DsRed transgenic line. It will also be possible to use *in vivo* imaging to study the kinetics of secretion within the affected cell networks. For example, the imaging system can be used to image GH-GFP secretion from cell clusters or single cells in response to an acute stimulus by fluorescence tracking or calcium imaging. This will provide

information on the activity and cell-cell communications of the remaining cells of the network.

6. A PRELIMINARY EXPLORATION OF NEW APPROACHES TO CONDITIONAL ALTERATION OF SOMATOTROPH FUNCTION *IN VIVO*

6.1 INTRODUCTION

The previous chapters describe the effects of somatotroph-targeted expression of the viral ion channel, ^{H37A}M2, on the structure and function of the anterior pituitary. However, the main disadvantage of using this approach is that the M2 channel is expressed in its active “open” form directly from the transgene. Rimantadine is required to block the channel and provide the conditional control that was the initial aim of this model. As has become apparent, this may be difficult to achieve without constant access to the cells, limiting it to *in vitro* uses. We therefore explored other strategies to conditionally alter GH cell function.

There are other possible ion channel strategies that could be exploited. These have previously been used to study the electrical properties of neurons. For example, receptor-mediated potassium channels have been used to suppress neuronal excitability in brain slices (Lechner et al., 2002) and *in vivo* (Gosgnach et al., 2006). The allatostatin G-protein coupled receptor of *Drosophila* can be coupled to endogenous inwardly rectifying potassium channels (Lechner et al., 2002). Treatment with allatostatin results in quick activation of the channels leading to hyperpolarisation of the cell membrane and in turn cell silencing. This effect is reversed when treatment is stopped. The main disadvantage of exploiting potassium channels is that the ion imbalance caused by expression/induction of potassium channels can result in apoptosis of the target

cell (Nadeau et al., 2000) and not cell silencing as desired. A second approach utilises the RASSL (Receptor Activated Solely by Synthetic Ligands) inhibitory G protein coupled receptor (GPCR). The endogenous inhibitory GPCR receptor is altered to confer insensitivity to the endogenous ligand to ensure the receptor is only activated by treatment with a synthetic form of the ligand (Coward et al., 1998). Treatment with the specific synthetic ligand activates the inhibitory pathway and silences the cell. This has been applied to opioid receptors *in vitro* (Coward et al., 1998).

The second ion channel selected to create an inducible transgenic mouse model of somatotroph silencing was the invertebrate Ivermectin-sensitive glutamate-gated chloride (GluCl) channel. Unlike the M2 channel, the Ivermectin-sensitive GluCl channel requires the drug, Ivermectin (IVM), to be present to initiate channel activity. Therefore, under physiological conditions in the absence of the drug the target cell will be unaffected. This approach should enable conditional silencing to alter the function of the GH cell population by addition, and subsequent removal, of IVM.

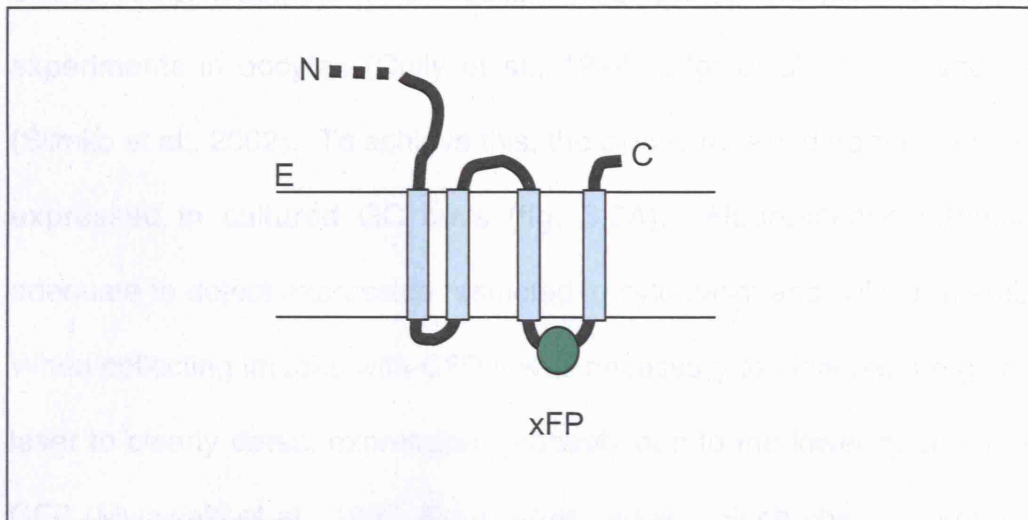
GluCl channels are part of the nicotinic receptor superfamily and are thought to share the common heteropentameric structure (Cully et al., 1994). They share structural homology with the inhibitory GABA- and glycine-gated chloride channels found in vertebrates. The α (GluCl α) and β (GluCl β) subunits of the GluCl channel were first cloned from *C. elegans* (Cully et al., 1994). Each of these subunits has been shown to form functional, homomeric channels when

individually expressed in *Xenopus laevis* oocytes. Further electrophysiological recordings have indicated that GluCl α channels are sensitive to IVM whereas those formed by GluCl β subunits confer glutamate sensitivity (Cully et al., 1994; Etter et al., 1996) suggesting that both may in fact be required to form functional channels. HA Lester (CIT, California) developed this as a method for conditional silencing, and he has kindly provided us with two constructs (GluCl α and β). These constructs were codon optimised (Slimko and Lester, 2003) to improve expression in mammalian cells and are fluorescently tagged with YFP (α) and CFP (β), located between the third and fourth transmembrane domains (figure 6.1). It has been shown that incorporation of the fluorescent tag does not alter function of the subunits. Additionally, the β subunit has been modified to increase selectivity for IVM so that it will bind IVM preferentially over glutamate (Li et al., 2002). Work done by Slimko *et al.* (2002) has shown that these constructs can be used *in vitro* to selectively silence electrical signalling from mammalian neurons. There were no published results for *in vivo* attempts to reproduce this when I began my project.

Somatotrophs are an excitatory population of cells. An electrical signal can be measured as movement of calcium ions in a resting state (Bonnetfont et al., 2000) and in response to the binding of GHRH (Cuttler et al., 1992) suggesting that it will be possible to silence cell function in a similar way to neurons. Also, mammals do not have GluCl channels and the concentration of IVM used is too low to trigger other mammalian glutamate-driven responses (Schinkel et al., 1994). However, it has been shown that dissociation of IVM from the channel is

6.2 RESULTS

Figure 6.1 Schematic diagram of a GluCl subunit containing a fluorescent tag (adapted from Slimko et al., 2002). E = extracellular domain, I = intracellular domain, xFP = fluorescent protein.



extremely slow (Slimko et al., 2002). This may prevent a rapid reversal of the Ivermectin-induced phenotype, although recovery may be possible through *de novo* channel synthesis *in vivo*.

The aim of the work described in this chapter was to test whether it would be useful to generate a transgenic mouse that could be used to reversibly silence somatotrophs *in vivo* where the ion channel would normally be closed, thus improving on the GH-M2 transgenic. A second, novel approach for expression of the two constructs with a single transgene containing a short linker peptide, 2A, was also attempted *in vitro* in this part of my work.

6.2 RESULTS

6.2.1 Initial GluCl-CMV transfections

The first step was to determine the cellular localisation and effect of expression of the GluCl channels in GH cells, in comparison to previous expression experiments in oocytes (Cully et al., 1994; Etter et al., 1996) and neurons (Slimko et al., 2002). To achieve this, the clones received from HA Lester were expressed in cultured GC cells (fig. 6.2A). Fluorescence intensity was adequate to detect expression restricted to cytoplasm and cellular membranes. When collecting images with CFP it was necessary to increase the gain on the laser to clearly detect expression, probably due to the lower quantum yield of CFP (Miyawaki et al., 1997; Rizzo et al., 2004). Such channel proteins, with membrane-spanning domains, may be expressed in the membranes of cellular compartments such as the endoplasmic reticulum and golgi bodies as they pass through the secretory pathway. Overexpression of the constructs in GC cells did appear to have an adverse affect on the state of the cells. Membrane blebbing was regularly observed. A clear example of this can be seen in figure 6.2Av and vi. Membrane blebbing is a morphological characteristic indicative of poor health and cell death (reviewed by Loo and Rillema, 1998). It was not possible to maintain stable cell lines expressing these constructs. It is not clear if this was a result of expressing GluCl channels with basal activity in the absence of IVM or the conditions of transfection.

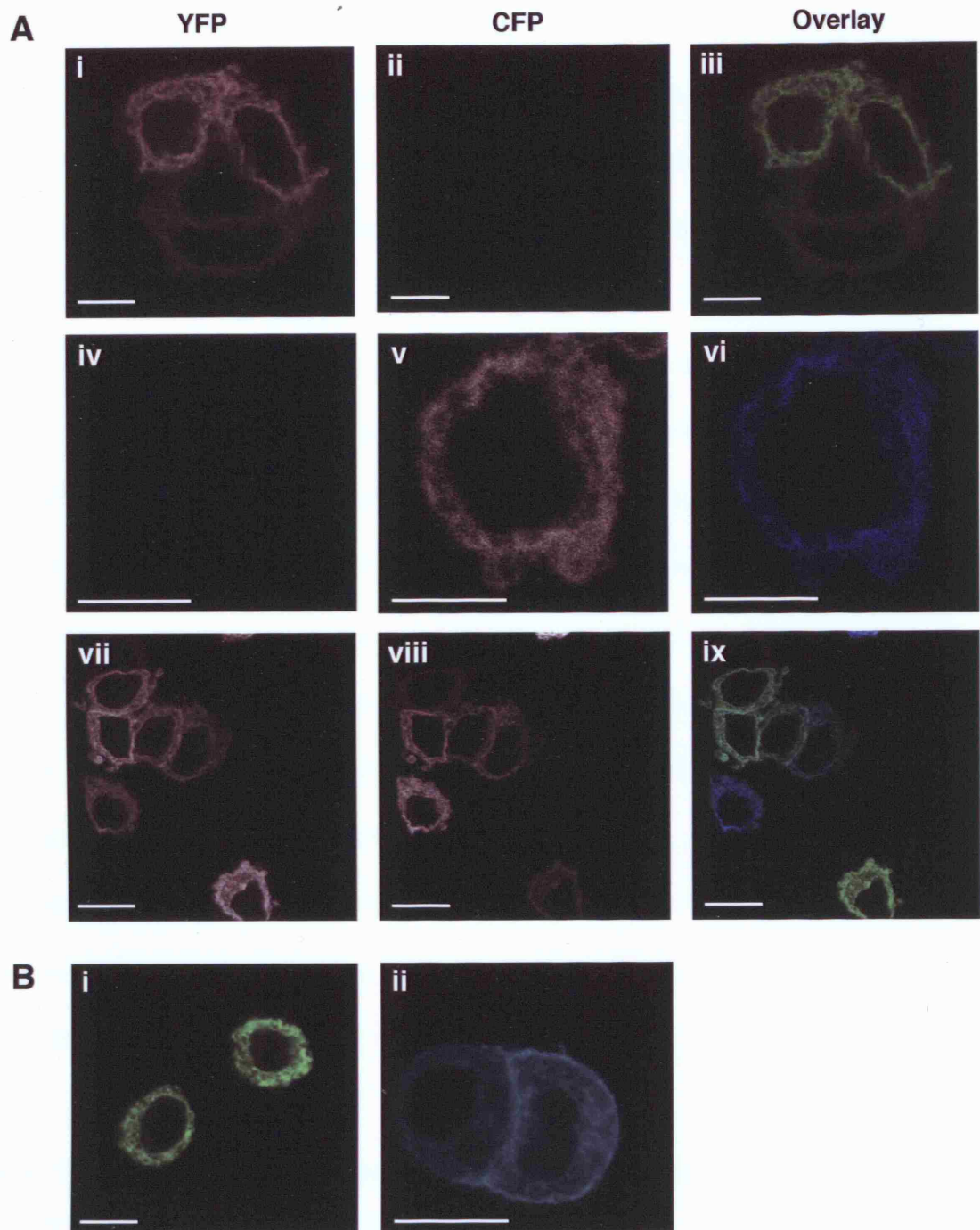


Figure 6.2 GC cells expressing CMV-driven GluCl α -YFP and GluCl β -CFP.

(A) Nucleofection with 2 μ g of DNA was used to transfect GC cells. 48hrs after transfection cells were fixed and expression imaged using confocal microscopy. Images i-iii show positive cells for a single transfection with GluCl α -YFP, iv-vi for a single transfection with GluCl β -CFP, and vii-ix a double transfection with both of these constructs. Scale bar equals 10 μ m.

(B) GC cells were transfected with (i) GluCl α -YFP, or (ii) GluCl α -YFP and GluCl β -CFP by nucleofection and cultured in growth medium containing 2 μ M Ivermectin. After 48 hours cells were fixed and imaged by confocal microscopy to look for changes to cell viability. Scale bar equals 10 μ m.

It was not yet clear from the expression results at this stage if the α and β subunits formed complete and functional channels when expressed either separately or together, as previously shown by expression in *Xenopus* oocytes (Cully et al., 1994) and neurons (Slimko et al., 2002) *in vitro*. To test this I studied the effect of Ivermectin treatment. Transfected cells were treated with 2 μ M Ivermectin for 48hours, as suggested by previous experiments from this lab. Preliminary data from confocal images of cells transfected with GluCl α -YFP (figure 6.2Bi), GluCl β -CFP (not shown) or both constructs (figure 6.2Bii) suggests that Ivermectin does not kill the cells in culture as there was no apparent cell loss in the presence of Ivermectin. The cell state appears similar to that of untreated transfected GC cells (fig 6.2A), and counts of transiently transfected cells indicate no difference in transfected cell number between treated and untreated cultures (table 6.1) suggesting that Ivermectin treatment does not induce cell death. Variability of transfection rate may affect numbers between experiments.

Table 6.1 Percentage of transfected cells in culture in the presence and absence of Ivermectin 48 hours after transfection

Construct	With Ivermectin	Without Ivermectin
GluCl α	82.0	70.9
GluCl β	30.4	22.1
GluCl α & GluCl β	79.3	64.7

6.2.2 hGH-GluCl constructs and analysis

In order to direct expression of the GluCl subunits to somatotrophs, a cloning strategy was designed to insert the sequence encoding the GluCl channel into the hGH LCR, previously used for transgenes such as GH-GFP (Magoulas et al., 2000) and GH-M2, which targets expression specifically to GH cells. The cloning strategy followed for GluCl α -YFP and β -CFP is shown in figure 6.3A and B, respectively. The final constructs were packaged into the hGH LCR cosmid and sequenced across the *MluI* insert boundaries to check orientation of the construct was correct.

These constructs were transiently transfected into GC cells to ensure that expression of these constructs occurred using the hGH LCR *in vitro*. Figure 6.4 shows GC cells transfected with the hGH-GluCl- α YFP and - β CFP constructs (fig. 6.4A and B). We decided to use a lipofection technique instead of nucleofection due to the size of the construct in the hope of achieving higher transfection efficiency. Other conditions were tried but resulted in either cell death or reduced transfection efficiency. The optimal conditions for transfections were found to be 2 μ g of DNA using Lipofectamine 2000 (3 μ l). The transfection efficiency and expression of the large GH-LCR driven constructs, as estimated from cell counts and confocal microscopy, are lower compared to the original constructs expressed from the CMV promoter. This may be expected due to the strong expression drive of the CMV promoter.

Figure 6.3 Cloning strategy for the GluCl constructs. A series of PCR amplifications and restriction enzyme digests were used to convert the promoter of the original constructs from CMV to the hGH LCR provided by the M2-GH cosmid. The final cosmid was purified using a salt gradient prior to microinjection and embryo transfer. Primer sequences are listed in materials and methods table 2.1.

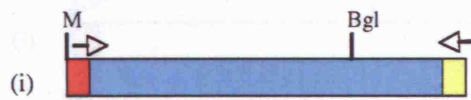
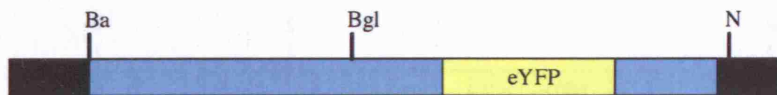
(A) GluCl α -YFP

(B) GluCl β -CFP

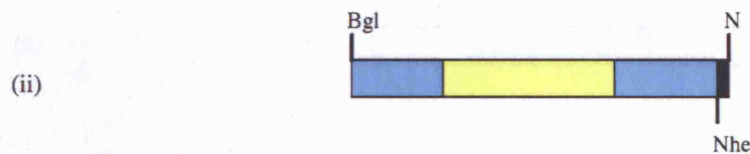
Restriction enzymes:

A – *Apa*I; Ba – *Bam*HI; Bgl – *Bgl*II; M – *Mlu*I; N – *Not*I; Nhe – *Nhe*I; S – *Sac*I

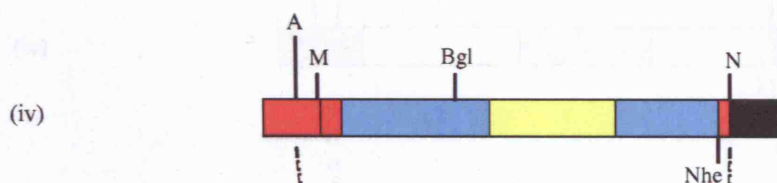
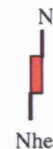
Original clone from Lester group:



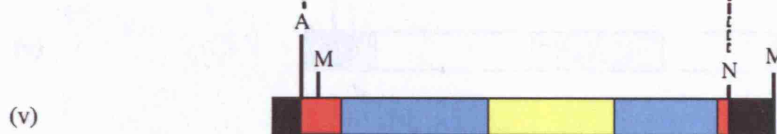
PCR amplification of Glu-YFP to generate a fusion of the GH 5' untranslated region with the coding region of GluCl α (primers glua α f and gluar)



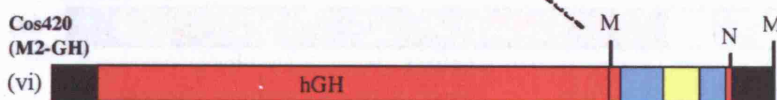
(iii) PCR amplification of p296 using primers NheBglGH (f) and T7 (r) to insert NheI site in the 3' untranslated region of GH



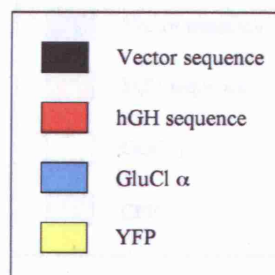
Ligation of fragments:
(i) *MluI*-*BglII*,
(ii) *BglII*-*NheI* and
(iii) *NheI*-*NotI*
into plasmid pBK-CMV



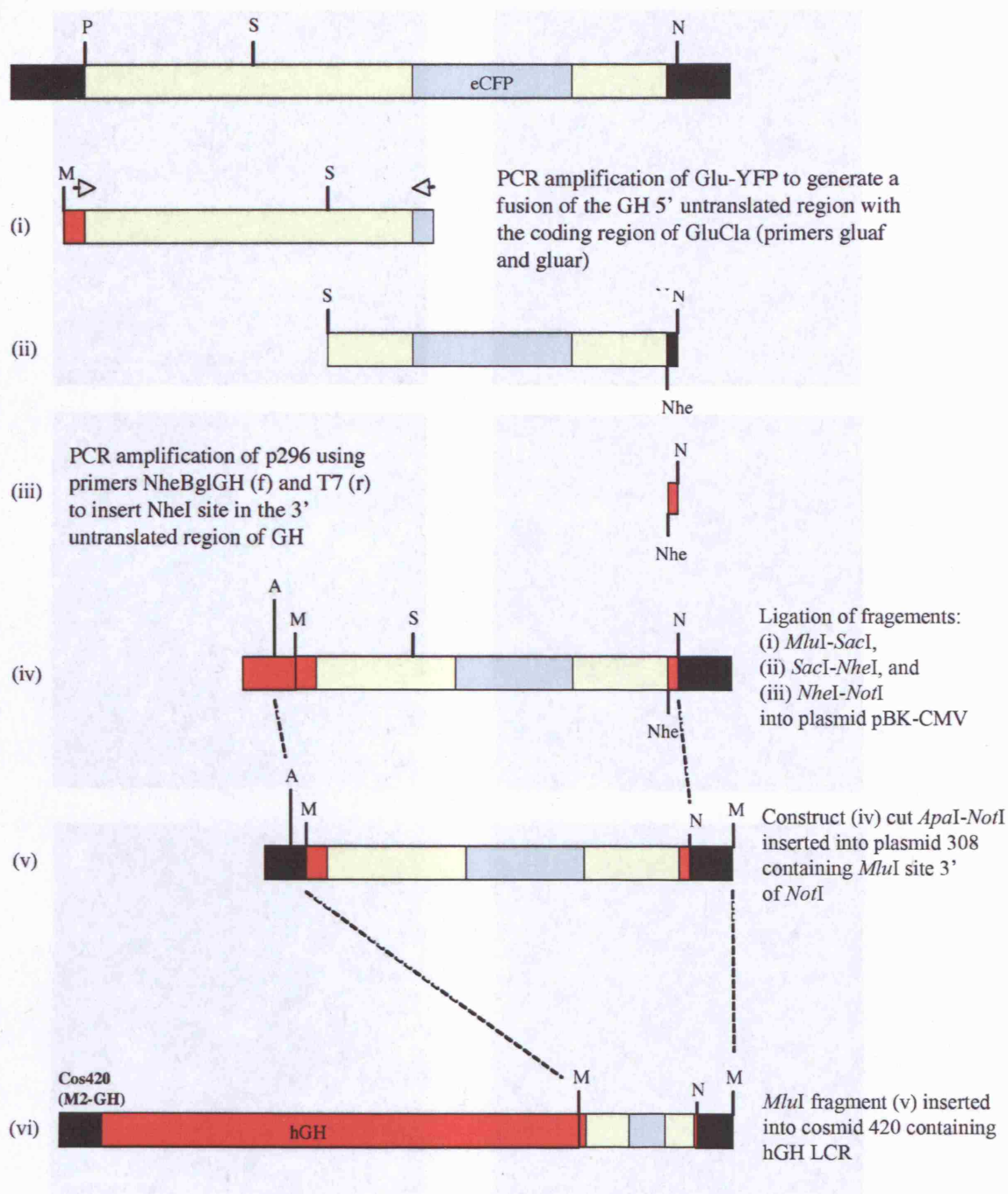
ApaI-*NotI* fragment from
step (iv) cloned into plasmid
308 to insert 3' *MluI* site.



MluI fragment (v) inserted
into cosmid 420 containing
hGH LCR



Original clone from Lester group:



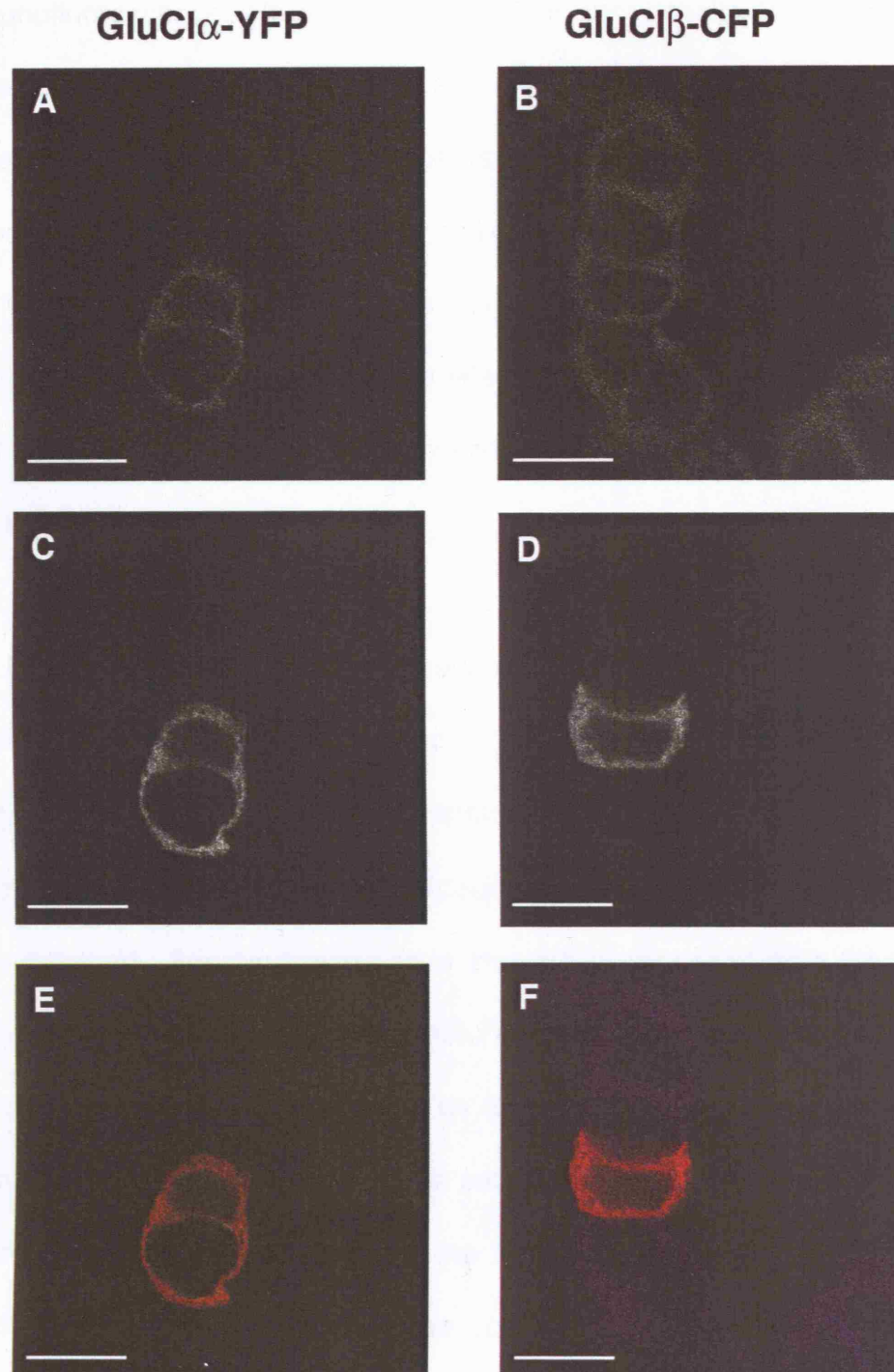


Figure 6.4 GC cells expressing hGH LCR driven constructs.

The hGH-GluCI- α YFP or hGH-GluCI- β CFP constructs (2 μ g) were transfected into GC cells using Lipofectamine 2000 (3 μ l). Immunofluorescence for YFP and CFP was performed using an anti-GFP antibody to detect expression at low levels and images taken using confocal microscopy. (A) Fluorescence from hGH-GluCI- α YFP transfection, (B) Fluorescence from hGH-GluCI- β CFP transfection, (C) Immunofluorescence for YFP, (D) Immunofluorescence for CFP, (E) and (F) Overlay of fluorescent protein and xFP immunofluorescence. Scale bar equals 10 μ m.

Immunofluorescence with an antibody to GFP, that detects both CFP and YFP proteins, was used to identify successfully transfected cells and localise the protein (figure 6.4C and D). It appears that channel protein expression was associated with cellular membranes and cytoplasm in GC cells (figure 6.4E and F). Membrane blebbing was not as obvious in cells transfected with the hGH constructs (figure 6.4) as those transfected with the CMV-driven constructs (figure 6.2) suggesting that this is an effect of overexpression of the constructs with the CMV promoter.

The final hGH constructs were linearised with *NotI* and purified using a salt gradient to prepare the DNA for oocyte microinjection to generate transgenic mice. 34 pups were born for the α construct and 49 pups for the β . Using PCR to genotype the offspring from pronuclear microinjections no transgenic animals were detected. Microinjections were stopped at this point as a mistake was discovered in the two constructs sent to us for incorporation in our sequences. Resequencing of the clones sent to us revealed that the β subunit still had the wildtype residue conferring glutamate sensitivity. Further injections could have been carried out in order to obtain the transgenic animals. However, at this point, since we had to remake the construct, we took the opportunity to investigate the use of a single construct to express the two subunits.

6.2.3 Expressing GluCl α and β from a single transgene

2A is a short 18 amino acid peptide sequence from the Picornavirus. It has been shown that cleavage of the picornavirus polyprotein is mediated by the 2A

peptide (Ryan et al., 1991), between the C-terminal proline-glycine of 2A and the N-terminal proline of the neighbouring peptide sequence, 2B (Donnelly et al., 2001) (see figure 6.5 below). The process by which this 'cleavage' occurs has been termed a ribosomal 'skip' as it is thought that it is not a proteolytic event that occurs post-translationally but a failure to form a peptide bond mediated by the ribosome during translation of the sequence (de Felipe et al., 2003). This 2A peptide has been shown to be useful for stoichiometric co-expression of two or more protein products (Chaplin et al., 1999; Fang et al., 2005), providing benefits over the use of an Internal Ribosome Entry Site (IRES) where expression of the second gene is generally substantially lower than the first, 5' gene (Mizuguchi et al., 2000). It was hoped that this technology could be used to successfully express GluCl α and - β from a single transgene in stoichiometrically equal amounts.

Figure 6.5 The 2A peptide sequence from foot and mouth disease virus.
Arrow denotes the point at which the ribosomal 'skip' occurs.

PESLVGSGLLNFDLLKLAGDVESNPG  P

A construct was made by Molly Strom, Molecular Neuroendocrinology, that expressed both the GluCl α and GluCl β subunits. This was achieved by inserting the 2A nucleotide sequence (corresponding to the peptide illustrated in figure 6.6A) from picornavirus between the GluCl β -CFP and GluCl α -YFP constructs. During translation of the 2A sequence a ribosomal 'skip' will occur

between the terminal glycine and proline of the linker sequence resulting in a separation of the α and β proteins. This results in addition of 18 amino acids to the N-terminus of the GluCl β and addition of a proline residue to the C-terminus of GluCl α . It is unknown what effect this will have on the function of the expressed proteins.

To test the efficiency of the 'skip' at the 2A sequence to produce separate GluCl α -YFP and β -CFP proteins a Western blot was used. NIH-3T3 cells were transfected with either the 2A-linker construct (GluCl-2A), GluCl α -YFP, or GluCl β -CFP using Lipofectamine 2000. NIH-3T3 cells were used instead of GC cells as they showed greater transfection efficiency and therefore produced a greater amount of protein for a Western blot. After 48 hours the cells were harvested with Laemmli buffer and heat denatured at 95°C before loading on 8% polyacrylamide gel. The gel was transferred to nitrocellulose membrane and incubated with anti-GFP antibody before being exposed to film. Figure 6.6B shows the resulting blot. The arrow marks the bands of the individual products. Unfortunately, due to the design of the 2A-linker construct there is only a small difference in the size of the two products following transcription and so the two run as one band. The size of this band was predicted to be approximately 78kDa but appears to run at a slightly smaller size (approx. 74kDa), possibly as a result of partial degradation of the proteins during the process of the experiment. Separation of the two components of the construct appears to be highly efficient as no band can be seen around 160kDa, the approximate size of

the unprocessed β -2A- α protein. It is possible, however, that the large 160KDa did not transfer efficiently to the blot.

A sample of cells from each β -2A- α transfection were fixed and imaged using a confocal microscope (fig. 6.7A). Expression of both YFP (i) and CFP (ii) can be detected in most positive cells, with some localisation to the cell membranes. This is most likely due to expression of the individual products, as shown by Western blot, rather than expression of the intact 2A protein. Protein localisation has yet to be assessed in GC cells at high resolution.

The function of these transfected cells was kindly tested by Guilherme Neves, Neurophysiology, by whole-cell patch clamp assay. Untransfected and transfected cells were treated with 1 μ M Ivermectin for 1 minute and membrane current conductance measured at a constant voltage of -60mV. There is no response to treatment in untransfected cells indicating that there is no endogenous response to the drug under these conditions (fig. 6.7Bi). In contrast a marked response, measured by change in membrane current (nA), in the presence of Ivermectin was detected in transfected cells (fig. 6.7Bii) indicating an outward flow of chloride ions along the artificially generated mem-

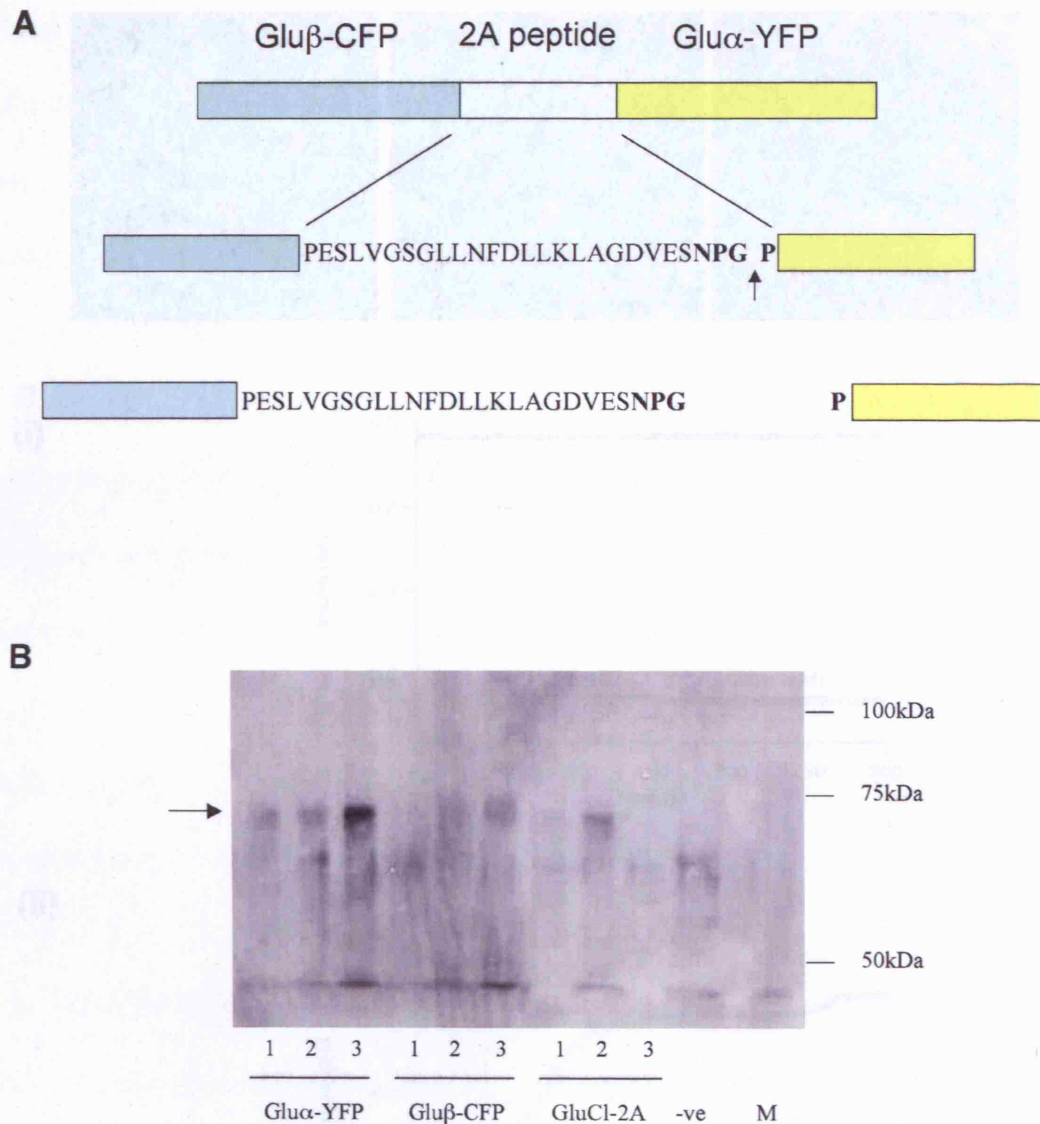


Figure 6.6 GluCl-2A protein expression and separation *in vitro*

(A) Schematic diagram of processing of the 2A-linker sequence. A ribosomal 'skip' occurs between the C-terminal glycine and proline, as indicated by the arrow, adding further amino acids to the processed peptides.

(B) The efficiency of the protein cleavage was tested using Western Blot. NIH-3T3 cells were transfected with GluCl α -YFP, GluCl β -CFP or GluCl-2A (each n=3) and then harvested 48hrs post-transfection. Heat-denatured samples were run on 8% polyacrylamide gels. The arrow marks the size of the cleaved products.

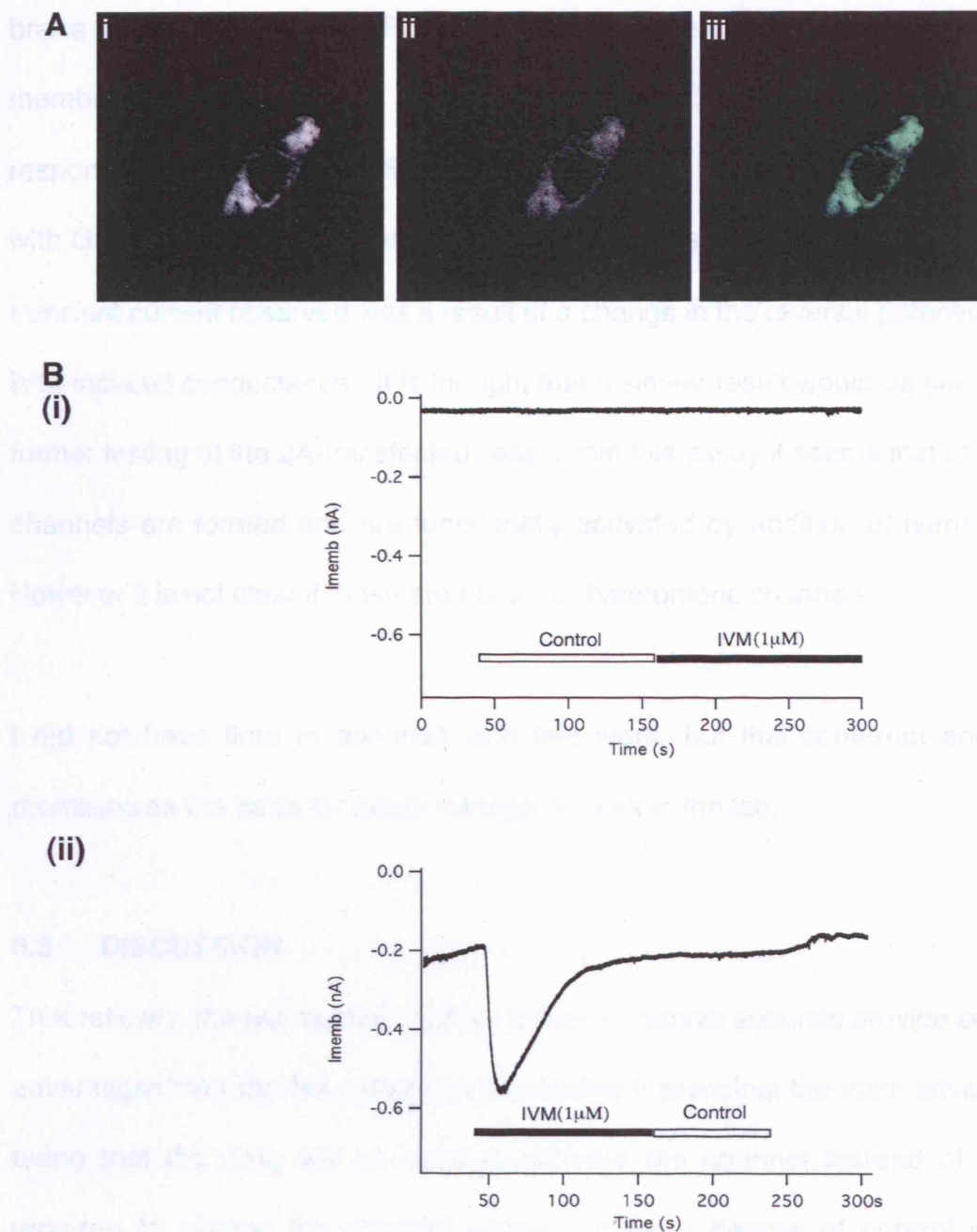


Figure 6.7 Functional analysis of NIH-3T3 cells transiently transfected with β -2A- α .

(A) NIH-3T3 cells were transfected with β -2A- α . Cellular expression of the β -2A- α proteins was imaged using confocal microscopy where YFP (i) and CFP (ii) were imaged sequentially and then overlaid (iii) for analysis.

(B) An example of whole cell patch clamp of untransfected (i) and β -2A- α transfected (ii) NIH-3T3 cells on addition of Ivermectin. Change in current across the plasma membrane was measured (nA).

brane potential gradient. Following the initial response to Ivermectin the membrane current appears to return to the prestimulation level. A similar response was observed by Slimko *et al.* (2002) in HEK 293 cells transfected with GluCl α . They performed further analysis of this effect, concluding that the transient current observed was a result of a change in the reversal potential with IVM-induced conductance. It is thought that a similar result would be seen with further testing of the 2A-transfected cells. From this assay it seems that chloride channels are formed and are functionally activated by addition of Ivermectin. However it is not clear if these are homo- or heteromeric channels.

I did not have time to continue with this work, but this construct appears promising as the basis for future transgenic work in the lab.

6.3 DISCUSSION

Theoretically, the Ivermectin-sensitive chloride channel subunits provide several advantages over the M2 system for somatotroph silencing; the main advantage being that the drug will be used to activate the channel instead of being required to silence the channel giving a greater degree of control of the phenotype. However, to date I have not been able to generate transgenic mice from the hGH-GluCl- α YFP and β CFP constructs. It is not clear why this should be the case. It is possible that it is a result of the larger size of the cosmid reducing the chances of its incorporation into the mouse genomic DNA. In generating the GH-M2 mouse lines over 100 pups were born before any founders were obtained. Experience of Procedural Services, who perform the

microinjections, is that a greater number of positive pups are born from a single round of injections when the construct is small. Thus, further oocyte microinjections will be required to attempt to obtain expression *in vivo*. However, further *in vitro* analysis is needed to study the function of the individual channel subunits.

In vitro expression of both CMV and hGH LCR driven GluCl- α YFP and GluCl- β CFP showed that these channel proteins were targeted to cell membranes, although some diffuse, cytoplasmic staining was also detectable. Treatment with Ivermectin *in vitro* did not appear to cause any adverse effects to the transfected cells. Ivermectin sensitivity is conferred by the α subunit of the GluCl channel (Cully et al., 1994; Etter et al., 1996) indicating that the GluCl- α YFP subunits could form an active, Ivermectin-responsive channel *in vitro*. This will be tested by patch-clamp analysis of transfected GC cells in the presence of glutamate or Ivermectin. If this is the case then it may only be necessary to make a single transgenic mouse line. Further optimisation of the transfection of GC cells with the hGH LCR constructs is required to enable transfected cells to be easily distinguished from untransfected cells in culture without the use of immunofluorescence, as it has not been possible to generate stable cell lines with these constructs.

Transfection of GC cells with both CMV GluCl- α YFP and GluCl- β CFP results in a mosaic of expression levels of the two constructs, which may reduce the ability of the cell to only form active heteromeric channels. Etter *et al.* (1997)

indicated that heteromeric channel expression is required as the β subunits influence the coupling of the α binding site to channel gating increasing sensitivity of the channel, despite the fact that functional homomeric channels have been studied (Cully et al., 1994; Etter et al., 1996). It is also likely that the levels of expression of the hGH-GluCl channels in transgenic animals will vary depending on copy number. It is unclear what effect this difference in expression will have in a transgenic animal expressing both channel constructs, although when maintaining the transgenic mice it will be possible to select the most appropriate lines for breeding. The ability to produce both the GluCl α -YFP and GluCl β -CFP constructs from the single 2A sequence, as indicated by Western blot analysis, should overcome problems of varying expression levels of the two channels. This should increase the chances that both proteins are expressed in the same cell in a 1:1 ratio in turn increasing the chance of formation of functional heteromeric channels. FRET (Fluorescence Resonance Energy Transfer) analysis could be used to study the formation of the channels. It is unclear if the channels formed following transfection with the 2A sequence are homo- or heteromeric at the magnification of imaging used. FRET will occur when the donor molecule (CFP) and the acceptor molecule (YFP) are less than 10nm apart and can be used to detect heteromeric channel formation and activity (Biskup et al., 2004; Hellwig et al., 2005).

The 2A sequence will also enable us to produce a single transgenic line that expresses both proteins rather than having to make and cross two separate mouse lines. Although separate mouse lines may provide a greater degree of

control of breeding and genetics, for example GH-GluCl α could be crossed to PRL-GluCl β allowing mammosomatotroph silencing, it would be time consuming. Decisions on this would be made after a successful proof of concept 2A study.

Complete sequencing of the 2A construct at a later stage in the project (molly Strom) revealed that the GluCl α and β constructs sent by Lester were in fact still in the glutamate sensitive form. This was not picked up at an earlier stage as previous sequencing for checking the cloning strategy was only performed across the cloning boundaries due to the large size of the constructs, as it was assumed that the sequence was correct. We have now introduced the point mutations required to reduce glutamate sensitivity. To enable broader use of these constructs in the future, for example for neuronal expression, it was necessary to reduce glutamate sensitivity of the subunit, as it is possible that endogenous glutamate could activate a response resulting in opening of the closed resting state of this channel. However, it is unclear if glutamate sensitivity is a true disadvantage. It is not known if a low level of glutamate activity would adversely affect somatotrophs as glutamate activation of the GluCl channels is thought to be rapid and reversible (Pemberton et al., 2001). Treatment with Ivermectin, with its extremely slow off-rate, may still be required to cause the long-term silencing of somatotrophs desired to study intercellular interactions and pituitary function.

Further work is required to test the advantages of using the GluCl-2A construct over the single hGH-GluCl constructs. Cloning of the GluCl-2A construct into the hGH LCR cosmid and transfection in to GC cells will indicate *in vitro* if it is possible to improve the low level of expression previously seen with the single constructs expressed from this cosmid compared to CMV constructs. Although low expression levels appear to result in fewer visible adverse affects *in vitro*, e.g. membrane blebbing, it has been shown by Slimko *et al.* (2004) that a low level of expression, detectable by *in situ* hybridisation but not fluorescence imaging, in neurons *in vivo* does not result in a significant modulation of neuron electrical activity or mouse behaviour when treated with Ivermectin. This low level of expression may be a result of the promoter used. It is hoped that using the hGH LCR will overcome this problem in the pituitary *in vivo* as is known from previous work (GH-GFP (Magoulas et al., 2000) and GH-M2 transgenics) that expression from this promoter is robust. Patch-clamp assays will be used to assess the functionality of the channels in the presence, and absence, of Ivermectin. It may also be beneficial to test the constructs in primary cultured anterior pituitary cells to study the expression of the constructs in a more physiologically relevant cell type prior to microinjection of the constructs.

There are other techniques that could be explored to provide conditional silencing of the somatotroph population. As an alternative to ion channel targeting in neurons it is possible to alter intracellular mechanisms, such as secretory vesicle exocytosis, to inactivate transmission of an action potential at the synapse in effect silencing a neuron population. One such mechanism

involves the use of small, inducible dimerizer molecules to bind to synaptobrevin or synaptophysin to prevent secretory vesicle fusion with the plasma membrane and, therefore, exocytosis (Karpova et al., 2005). This system, termed MIST (Molecular System for inducible and reversible Inactivation of Synaptic Transmission), exploits the rapamycin-inducible activity of FKBP. Binding of rapamycin, and its non-toxic derivatives, to FKBP results in a conformational change that exposes the molecular binding sites of FKBP to enable binding of FRB (FKBP/rapamycin binding) domains (reviewed by Crabtree and Schreiber, 1996; Rivera, 1998). This method has previously been applied to the analysis of signal transduction pathways (Spencer et al., 1993; Belshaw et al., 1996) and conditional cell ablation (Mallet et al., 2002). Karpova *et al.* created FKBP/FRB fusions with synaptobrevin and synaptophysin. Upon treatment with a non-toxic rapamycin derivative inactivation of synaptic transmission was rapidly induced. This process was reversible both *in vitro* and *in vivo* upon removal of the drug. Unlike ion channel transgenes, MISTs do not rely on the modification of cell excitability.

7. GENERAL DISCUSSION

The overall aim of this project was to develop a mouse model of reversible GH deficiency to study the interactions of the somatotroph and lactotroph populations. I have successfully generated three fertile GH-M2 mouse lines and this thesis discusses the characterisation of these mice, and the novel discoveries about cell organisation revealed in these mice. However, it has not been possible to reverse the effect of channel activity *in vivo* with Rimantadine treatment.

The severity of the phenotype induced by transgene expression varies with transgene copy number. All three lines have a significantly decreased pituitary GH content from 14 days. Despite this the low, and to some extent the medium, copy line show an increase in pituitary GH content by 6 weeks of age in a pattern that follows that of non-transgenic mice. A similar increase in pituitary GH content with age is observed in the GH deficient Tgr rat (Flavell et al., 1996). 2-photon imaging of GH-M2 pituitaries to 40 days suggests an increase in somatotroph number with some remaining organisation of the network with age in low and medium copy line male mice. This may indicate that a degree of expansion of the somatotroph population can occur in response to the endogenous stimuli during puberty, although the level of disruption of the network appears to correlate with the decreased pituitary GH content of these GH-M2 lines. The GHRH-M2 mouse (Le Tissier et al., 2005) is dwarf and severely growth hormone deficient. 2-photon imaging of the GHRH-

M2/GH-GFP pituitary reveals that the GH cells are isolated and dispersed with no clear remaining network structure suggesting that GHRH is a necessary cue for somatotroph network formation. This contrasts to the GH-M2 mice where small clusters and strings are detectable suggesting that disruption of somatotroph function per se does not impair formation of network structures.

A compromised ability of the medium and high copy line mice to respond to GHRH and GH secretagogues would be expected, given their low GH content. The inability of low copy line male mice to mount the expected response to GHRH and GH secretagogues is more surprising. One possibility for this is that the pool of pituitary GH measurable by RIA cannot be secreted following acute stimulation. It is likely that as M2 channel activity increases in the cell it will alter the ion balance within the cell and across the cell membranes in the absence of Rimantadine, as shown by *in vitro* expression of ^{H37A}M2 in GC cells (Le Tissier et al., 2005). This may be sufficient to inhibit secretory vesicle exocytosis as the function of voltage-gated calcium channels may be disrupted. This effect is likely to be mechanism-independent as a deficient response is observed to both GHRH and GHRP-6. In turn this may impact on the somatotroph network as a whole since synchronised calcium spikes due to electrical activity have been recorded both spontaneously (Guerineau et al., 1998) and in response to GHRH (Kwiecien et al., 1997; Bonnefont et al., 2005). Silencing of voltage-gated calcium channels and disruption of the somatotroph network may prevent this synchronicity. This may affect both the secretory response to acute stimulation and the pattern of GH secretion, possibly

removing the pulsatility of secretion that is most obvious in male rodents (Tannenbaum and Martin, 1976; Clark et al., 1986).

It has also been reported that M2 can disrupt the Golgi network (Henkel and Weisz, 1998; Henkel et al., 2000). This would prevent the cell from efficiently processing molecules through the secretory pathway and may lead to accumulation of molecules in the cytoplasm. It is also possible that following such damage to the secretory pathway, and vesicle storage, that some GH is constitutively secreted. This could enable the low and medium copy line mice to maintain growth and plasma IGF-1 levels. The decline in pituitary GH content that begins to occur by 100 days of age may be a result of ablation of these cells with disrupted function and a reduced ability of the pituitary to replace the ablated GH cells. TUNEL staining may reveal an increase in cell death with age and BrdU incorporation could indicate the ability of the remaining cells to replace those ablated. Performing these studies in aged GH-M2 mice treated with a long-acting form of GHRH may suggest if a decrease in stimulatory drive with age is contributing to the observed impact of M2 activity.

Growth of low copy line mice is equivalent to non-transgenic mice whereas male medium copy line mice show a slower rate of body weight gain post-weaning and have a reduced body length. It is possibly that this is a direct result of the significantly reduced pituitary GH content of the medium copy mice, although a similar significant effect on growth is not observed in female medium copy line mice. Alternatively, if M2 channel activity is altering the response to

GHRH and secretagogues it is possible that the pulsatile pattern of secretion of GH is disrupted. The effect this has on growth would be most pronounced in male mice and may account for the significant difference in body weight gain and body length observed in the medium copy line male mice.

Other induced GH deficient models such as the dwarf mice generated by expression of thymidine kinase (Borrelli et al., 1989) and diphtheria toxin (Behringer et al., 1988) were severely growth retarded from an early age with a dramatic reduction in the number of somatotrophs remaining. The high copy line mice exhibit a GH-deficient phenotype similar to these models, with an undetectable level of pituitary GH and plasma IGF-1 by RIA and a dramatic ablation of somatotrophs. It is possible that the difference observed in the growth and GH phenotypes between the medium and high copy line mice is due to the threshold for M2 expression being reached in the high copy line mice where M2 expression and activity is sufficiently high to induce rapid somatotroph ablation.

These findings suggest that an accumulation of M2 channels in the GH cell is required before ablation occurs. If more M2 channel is required it is possible that there are several steps to M2 disruption of cellular function, including one of cell silencing reducing the somatotroph response to GHRP-6 and GHRH. This is in contrast to the diphtheria toxin ablation model (Behringer et al., 1988) where an increase of pituitary GH content and somatotroph population expansion did not occur. These mice are dwarf from young age as it is believed

that only one molecule of diphtheria toxin is required to ablate a cell (Yamaizumi et al., 1978).

Low and medium copy line mice can maintain plasma IGF-1 levels similar to their non-transgenic littermates indicating that a proportion of somatotrophs must maintain their ability to secrete GH to support the GH-IGF-1 axis. Other factors such as food intake (McKnight and Goddard, 1989) may be able to compensate for the decrease in GH secretion. A decrease in pituitary GH content and cell number usually results in a decrease of the level of circulating IGF-1 affecting growth and body composition (Woodall et al., 1991; Donahue and Beamer, 1993; Ezzat et al., 2006b; Liao et al., 2006). It appears that the threshold pituitary GH content and cell number for maintaining IGF-1 levels falls between that of the medium and high copy lines, as medium copy line mice can maintain a relatively normal growth rate whereas high copy line mice are severely growth retarded. It is possible that the remaining GH cells in the medium copy line pituitary have an increased rate of GH production and secretion to maintain IGF-1 levels, and therefore growth, due to an increase in GHRH stimulation induced by the GH deficiency (Frohman et al., 1989; Phelps et al., 1993; Peng et al., 2001). This would further decrease the measurable pituitary GH content. It would be interesting to sample plasma GH in the low and medium copy lines. Although limited by the size of mice and constraints of the licence it is possible to take blood samples over a period of hours in normal mice. It would be interesting to ascertain the pattern of GH secretion in these transgenic mice. Another alternative is to use northern blot analysis of liver GH-

regulated genes, e.g. MUP and PRLR, to indirectly assess the alteration of GH secretion in male and female mice, as shown by analysis of the somatostatin knockout mouse (Low et al., 2001).

The development of an *in vivo* real-time imaging system by the Mollard group in Montpellier provides the possibility for *in vivo* studies of GH secretion. Using GH-M2 mice crossed to the GH-GFP strain it will be possible to study the secretion from cell clusters and single cells of the disrupted somatotroph network in response to acute stimulation in anaesthetised mice. It would also be possible to study the excitatory response to stimulation with GHRH and GHRP-6 using this system with calcium imaging, previously used in study of pituitary slices (Guerineau et al., 1998; Bonnefont et al., 2005). This may reveal whether M2 activity is silencing the cells prior to ablation. Real-time imaging of PRL gene promoter activity, and factors regulating activity, is possible following expression of an adenoviral vector containing the luciferase reporter gene under the regulation of the PRL promoter (Stirland et al., 2003; Friedrichsen et al., 2006). By imaging changes in luciferase luminescence following stimulation, the relative effects of factors such as forskolin, TRH and TNF- α on transcriptional activity have been assessed. It may be interesting to apply this technique to study the effect that M2 activity has on GH synthesis in GC cells stably transfected with M2 and primary cultured anterior pituitary cells from the GH-M2 lines.

Analysis of other pituitary hormones revealed a variable effect on the pituitary PRL content between the three lines. Treatment with estrogen for two weeks led to a significant increase in pituitary PRL content in low and medium copy line male mice suggesting that the responsiveness of lactotrophs, mammosomatotrophs and somatotrophs to estrogen is maintained (Lieberman et al., 1981; Boockfor et al., 1986; Gonzalez-Parra et al., 1996; Goth et al., 1996) although to a lesser extent in medium copy line mice. Low and medium copy line female mice can be used as breeders suggesting that the PRL deficiency is not sufficient to severely affect fertility. This is in contrast to the complete deficiency observed in PRL and PRL receptor null mutant mice where PRL has been shown to be essential for female reproduction and lactation (Horseman et al., 1997; Ormandy et al., 1997). Surprisingly, an effect was also detected on the pituitary TSH content of medium and high copy line mice. Although it is possible that this resulted from misexpression of the transgene this is unlikely to have occurred in both of these GH-M2 lines. If lactotrophs and thyrotrophs have been directly affected by equivalent M2 expression then it would stand to reason that the degree to which these cell types were affected would be similar to the extent of GH deficiency in the three lines. This was not observed. In the following proposed models I have tried to integrate my results in terms of how M2 could affect the Pit-1 cell lineage:

1. Low level transgene expression in progenitor/precursor cells
2. Somatotroph-specific transgene expression alters lineage progression
3. Accelerated turnover and progenitor cell depletion with age

It is believed that GH and PRL cells differentiate from a common precursor cell, possibly the mammosomatotrophs (Nikitovitch-Winer et al., 1987; Borrelli et al., 1989). Although thyrotrophs also differentiate from a Pit-1 expressing cell, as illustrated by their absence in the Snell dwarf mouse (Roux et al., 1982; Li et al., 1990), they are not thought to arise from the same precursor as somatotrophs and lactotrophs. *In situ* hybridisation studies of the embryonic pituitary (Japon et al., 1994) indicated that TSH expression could be detected at e14.5 whereas simultaneous expression of GH and PRL occurred at e15.5. This, along with the existence of mammosomatotrophs, led to the theory of two divisions of the Pit-1 lineage and is summarised in figure 7.1. However, this may not necessarily be the case and the three cell types may differentiate directly from one precursor cell, as suggested in figure 7.2.

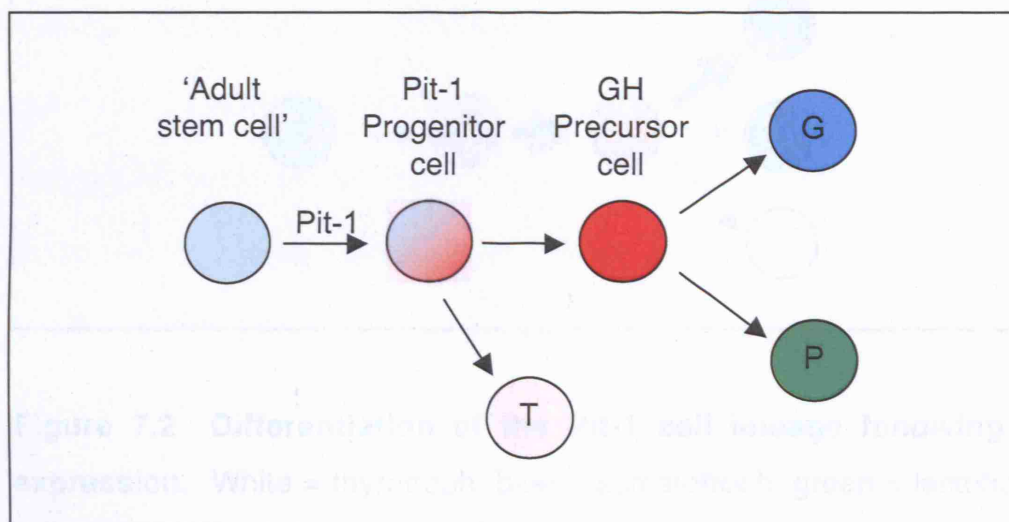


Figure 7.1 Schematic diagram of differentiation of the Pit-1 cell lineage.

T = thyrotroph, G = somatotroph, P = lactotroph

Model 1:

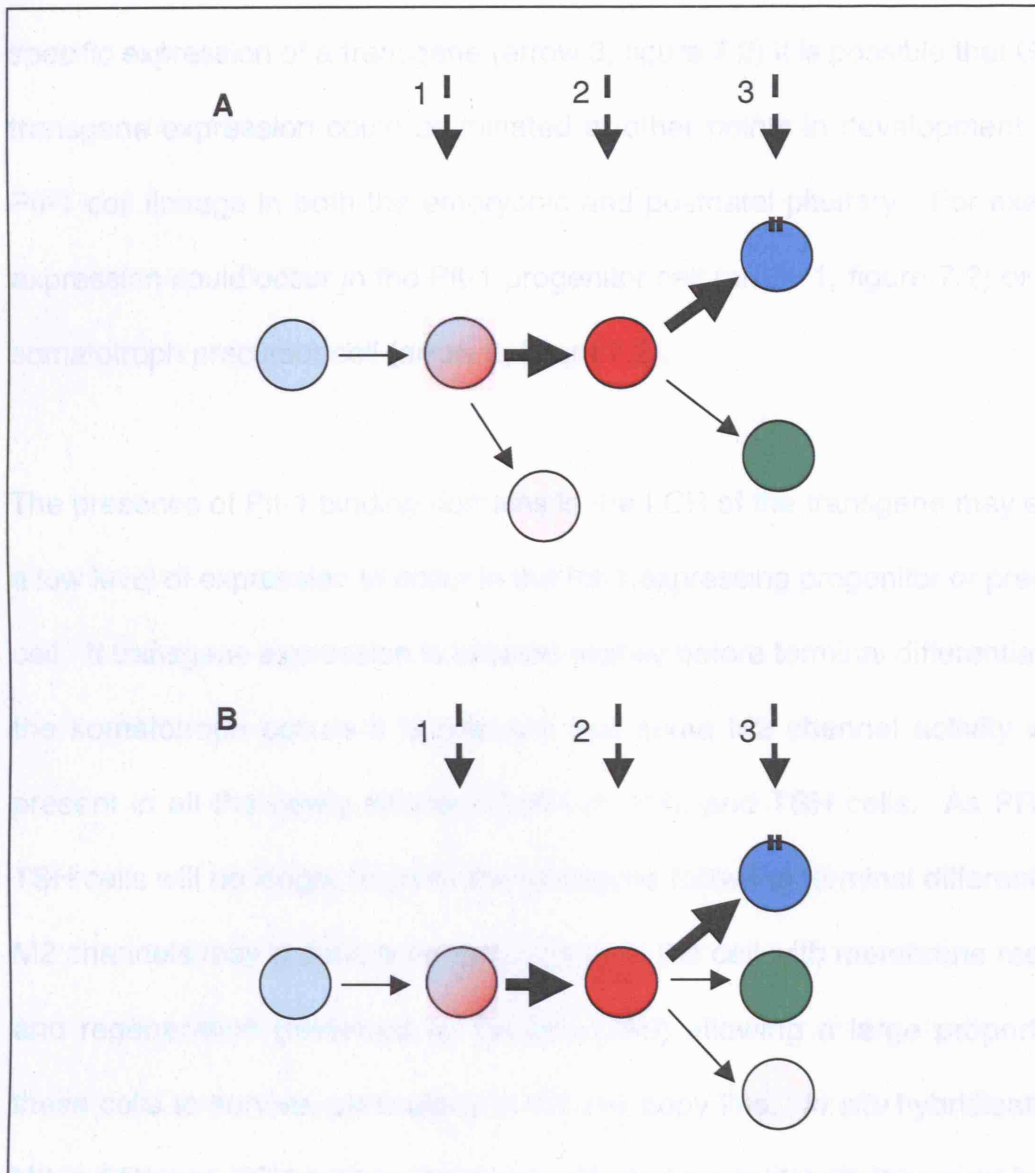


Figure 7.2 Differentiation of the Pit-1 cell lineage following GH-M2 expression. White = thyrotroph, blue = somatotroph, green = lactotroph. A - Separate thyrotroph precursor cell. B - Pit-1 cell lineage shares a common precursor cell. Arrows (dashed) and numbers denote point at which transgene expression could be initiated.

Model 1:

Although previous studies with the hGH LCR have indicated somatotroph-specific expression of a transgene (arrow 3, figure 7.2) it is possible that GH-M2 transgene expression could be initiated at other points in development of the Pit-1 cell lineage in both the embryonic and postnatal pituitary. For example, expression could occur in the Pit-1 progenitor cell (arrow 1, figure 7.2) or in the somatotroph precursor cell (arrow 2, figure 7.2).

The presence of Pit-1 binding domains in the LCR of the transgene may enable a low level of expression to occur in the Pit-1 expressing progenitor or precursor cell. If transgene expression is initiated slightly before terminal differentiation of the somatotroph occurs it is possible that some M2 channel activity will be present in all the newly differentiated GH, PRL and TSH cells. As PRL and TSH cells will no longer express the transgene following terminal differentiation, M2 channels may gradually be removed from the cell with membrane recycling and regeneration (reviewed by Brown, 1989) allowing a large proportion of these cells to survive, particularly in the low copy line. *In situ* hybridisation for M2 in PRL and TSH cells in the embryonic and postnatal pituitary may indicate if this is the case.

If thyrotrophs differentiate to form a separate lineage to somatotrophs and lactotrophs (figure 7.2A) a low level of transgene expression prior to the differentiated somatotroph may only affect the thyrotroph population if it was the

pluripotent precursor that was affected. However, if the three lineages originate from a single precursor (figure 7.2B) then a low level of transgene expression in either the progenitor or precursor cell could impact on the thyrotroph population. In the low copy line there is no significant difference in the pituitary TSH content at the ages studied suggesting that it is not the progenitor cell that is affected. A TSH deficiency is detectable from 14 days in the medium copy line. This may indicate that it is in fact a common precursor for the three lineages that is expressing a low level of the transgene.

Model 2:

The GH deficiency that results from GH-M2 expression in the somatotroph (arrow 3, figure 7.2) and the subsequent M2 channel activity is likely to lead to an increase in GHRH production and secretion, as observed in other GH deficient mouse models such as the Ames dwarf mouse (Phelps et al., 1993), *little* mouse (Frohman et al., 1989; Lin et al., 1993) and GH receptor knockout mouse (Peng et al., 2001). In turn this will increase the drive (bold arrows, figure 7.2) to synthesise more GH and increase GH cell number by proliferation of the existing population or differentiation of precursor cells. This may have the effect of reducing the drive or pool of available progenitor cells to produce PRL and TSH cells resulting in an apparent decrease in the numbers of these cells in the postnatal pituitary and a subsequent decrease in the measurable pituitary PRL and TSH contents. Treatment of low and medium copy line male mice with estrogen induced a small but significant increase in pituitary PRL content indicating that the lactotroph population is still responsive to this stimulus.

However, it is unclear if this is a result of increased PRL synthesis, an increase in PRL cell number, or both.

It may be interesting to image the effect of blocking the GHRH drive on the GH, PRL and TSH cell populations of the GH-M2 mice. If a strong drive towards the GH cell population is reducing the drive or depleting the pool of precursor cells available for lactotroph and thyrotroph differentiation it is possible that removing this drive, for example by crossing the GH-M2 mouse to a GHRH deficient mouse, will allow expansion of the other cell lineages. In the GHRH knockout mouse (Alba and Salvatori, 2004) there is no change in the pituitary PRL content despite the GH deficiency that results. In the GHRH-M2 mouse (Le Tissier et al., 2005) a reduction in pituitary PRL content is observed with the severe GH deficiency, however.

Model 3:

It is unclear whether the precursor cells that exist in the postnatal pituitary arise from stem cells or a pool of pluripotent progenitor cells that have already undergone a degree of differentiation (Chen et al., 2005; Chen et al., 2006). If a pool of stem cells exists these will continue to regenerate with every differentiation step that occurs and will not be depleted with age. On the other hand, if it is a pool of pluripotent progenitor cells that gives rise to newly differentiated somatotrophs, lactotrophs and thyrotrophs postnatally then a gradual decrease in the number of progenitors in the pituitary will occur with age as these cells do not have the ability to regenerate themselves and maintain a

constant pool of progenitors for differentiation. In the medium copy GH-M2 mouse line it appears that the effects of GH-M2 expression become more pronounced with age. A similar effect may be observed in aged low copy line mice. This may be because the increased drive to reduce the M2-induced GH deficiency depletes the progenitor cell pool with age. This in turn will be reflected in the pituitary PRL and TSH contents, as progenitor cells will be unavailable for differentiation to these cell populations. Immunohistochemistry for Pit-1 and Sox-2 (Komitova and Eriksson, 2004) in young and old GH-M2 mice may indicate if the progenitor/precursor cell population is subjected to an increased drive in young animals and subsequently depleted with age.

The high level of M2 expression in the high copy line may increase the speed/intensity with which the above models occur in the high copy line pituitary resulting in the severe phenotype. Although misexpression of the transgene cannot be discounted, it is also possible that the effect on pituitary PRL, TSH and LH content and cell number in the high copy line mice is a bystander effect of GH ablation. This was observed in the high copy lines of the hGH exon3 deletion mouse model (McGuinness et al., 2003), where a decreased number of lactotrophs, corticotrophs and gonadotrophs was attributed to an increase in macrophages imaged within the anterior pituitary. Electron microscopy of the pituitaries of the three lines may provide indications for such a mechanism in the GH-M2 mouse.

Further characterisation of the GH-M2 transgenic mice will include completion of the somatotroph network imaging and imaging analysis of the lactotroph network, using the Prl-DsRed model, to study the disruption and interaction of the two networks as the GH deficiency increases in severity. It will also be interesting to assess the proliferative ability of the remaining somatotrophs in the GH-M2 pituitary as well as the degree of cell death in the three lines. Additionally, low and medium copy GH-M2 male mice crossed to GH-GFP are to be sent to Montpellier for functional *in vivo* imaging analysis of secretion.

It has not been possible to reverse the GH deficient phenotype of the GH-M2 mice with Rimantadine treatment. A second model for temporal control of somatotroph silencing was therefore also explored. The Ivermectin-sensitive glutamate gated chloride channels require Ivermectin to be present to initiate channel activity (Slimko et al., 2002). Ivermectin acts rapidly to allow chloride ions to flow along their concentration gradient leading to hyperpolarisation and silencing of the cell. Transgenes have been generated which express the GluCl subunits α and β (Slimko et al., 2002; Slimko and Lester, 2003) from the hGH LCR. A low level of expression of these constructs *in vitro* has limited electrophysiological analysis with Ivermectin. Cells transfected with a single transgene construct with the ability to express both of the GluCl subunits in stoichiometric quantities were responsive to treatment with Ivermectin. Further analysis of the Ivermectin-sensitive GluCl channels and development of a transgenic mouse model may provide an alternative to expression of the M2 channel. Recent attempts by other groups to generate this transgenic model for

silencing of neurons had not been successful (Slimko et al., 2004). Direct injection of two adenovirus vectors (GluCl α and β) into the mouse brain (striatum), however, has recently enabled the study of Ivermectin-gated chloride channels as a system for selective neuron silencing and its effects on animal behaviour (Lerchner et al., 2007). Coexpression of the two subunits leads to Ivermectin-dependent silencing in positive neurons. Following treatment of the mice with amphetamine, strong rotational activity was observed to the side of subunit injection compared to control mice suggesting that neuronal activity had been compromised. Further refinement of this model is required to generate transgenic mice with cell-specific expression of the subunits.

The use of M2 still provides a good basis for study of the effects of disruption of a specific cell population. In order to gain conditional, temporal control of M2 expression a second mouse model is being developed. Control will be achieved through the use of a Tet-controlled M2 transgene that also contains the fluorescent tag cerulean (Rizzo et al., 2004). Addition of this fluorescent tag does not appear to adversely affect channel activity as *in vitro* studies of transfected cells show that cell death of M2 positive cells occurs and that this is reversible with addition of Rimantadine. By crossing these mice to those expressing a specific tetracycline-controlled transactivator protein (tTA or rtTA) it will be possible to use tetracycline treatment to inhibit or induce transcription in a specific cell type. For example, it will be possible to cross Tet-M2 mice to lines expressing rtTA in GH cells or tTA in PRL cells (Roh et al., 2001). A second level of control could be introduced by crossing the Tet-M2 mouse to a

Rosa26 floxed rtTA (Belteki et al., 2005; Yu et al., 2005) and a Cre line such as GH-Cre (Luque et al., 2007) or PRL-Cre. It will, therefore, be possible to induce M2 expression in cells that express, or have once expressed, Cre recombinase. This will enable the study of a specific cell lineage at different developmental ages and under different physiological conditions.

Work by Luque *et al.* (2007) using the GH-Cre mouse indicates that somatotrophs and the majority of lactotrophs form separate and distinct cell lineages in the developing and adult pituitary. This model and conclusions from the work presented in this thesis suggest that the relative fluctuations seen in cell type and number in the adult pituitary may be a result of alterations to the factors influencing differentiation and proliferation of the specific cell types and not transdifferentiation as previously proposed (Stefaneanu et al., 1992; Vidal et al., 2001). For example, in a normally functioning pituitary differentiation of progenitor/precursor cells maintains the balance of cells within the gland. When this balance is disrupted, in this case by removal of somatotrophs, the system must act to redress the balance, in turn leading to a depletion of other related cell types such as lactotrophs and thyrotrophs. Further work using the GH-M2 and Tet-On M2 models is required to address this issue in the mouse pituitary.

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